# CHARACTERISATION OF HUMAN CD5 B CELLS

by

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*to my mum and dad*

*If there are bounds to any man*

*Save those himself has set*

*To far horizons they're postponed*

*And none have seen them yet*

*And if most men are close curtailed*

*And keep a petty groove*

*'Tis their own sloth that is to blame*

*Their powers they will not prove*

***Sorley MacLean***

***"The Cuillin", 1939***

# ABSTRACT

The human 67kDa glycoprotein termed CD5, is equivalent to Ly1 (B-1) in the mouse. This molecule was, originally, described as a pan-T cell marker.

Evidence has been accumulating, suggesting that the B cells carrying this marker, would play a key role as producers of antibodies to self constituents.

These studies were initiated, to clarify the repertoires of human CD5 B cells.

CD5 molecules were found to be barely detectable on a minority of circulating B lymphocytes. A number of techniques improved the sensitivity of their detection and it was established that the blood of some patients with autoimmune disease, may, in fact, have a higher number of this subpopulation of B cells.

Furthermore, chronic lymphocytic leukemia B cells, expressing CD5, were shown to produce auto and polyreactive antibodies.

Since it was found that a high proportion of B cells, early in development, express CD5, clones were established by Epstein-Barr virus transformation of CD5+ and CD5- cord blood B lymphocytes, separated by flow cytometry. IgM from many of both CD5+ and CD5- clones reacted with a variety of autoantigens.

Examination for expression of cross-reactive idiotypes, associated with defined VH and Vκ subgroups, revealed that auto and polyreactivity does not appear to be the property of any particular V gene family. Indeed, IgM produced by both subsets of cord blood B cells, were found with equal frequencies of expression of VHI and VHIII associated idiotopes.

In contrast, a higher frequency of a VκIIIb subgroup associated idiotope, the phenotypic marker for the Vκ325 germline gene, was found in the CD5+ clones and of particular interest was the restricted association of the CD5 B cells with a VHIV subfamily.

In conclusion, differences may exist in the expression of certain germline genes between CD5+ and CD5- cord blood B cells and may indicate an expansion of CD5 B cells within the fetal environment.

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# ABBREVIATIONS

| | |
|---|---|
| **Ab** | antibody |
| **Ag** | antigen |
| **ANA** | anti-nuclear antigen |
| **ARC** | antigen responsive cells |
| **APC** | antigen presenting cells |
| | |
| **(Br)MRBC** | (bromelain treated) mouse red blood cells |
| **BSA** | bovine serum albumin |
| **BSS** | buffered salt solution |
| | |
| **CARD** | cardiolipin |
| **CD** | cluster differentiation |
| **CD5+** | CD5 positive |
| **CD5-** | CD5 negative |
| **CDR(s)** | complementarity determining region(s) |
| **CLL** | chronic lymphocytic leukemia |
| **CNBr** | cyanogen bromide |
| **COLL I/II** | collagen types I and II |
| **CRI** | cross reactive idiotope/idiotype |
| **CYTO** | cytokeratin |
| | |
| **D** | diversity gene segment of immunoglobulin |
| **DAB** | 3'3' diaminobenzidine tetrachloride |
| **DABCO** | diazabicyclo-octane |
| **DAG** | diacyl glycerol |
| **DMSO** | dimethyl sulphoxide |
| **DNA** | deoxy-ribonucleic acid |
| **dsDNA** | double-stranded deoxy-ribonucleic acid |
| **ssDNA** | single-stranded deoxy-ribonucleic acid |
| | |
| **EBNA** | Epstein-Barr nuclear antigen |
| **EBV** | Epstein Barr virus |
| **ELISA** | enzyme linked immunosorbent assay |
| | |
| **FcR** | receptor for the Fc region of immunoglobulin |
| **(HI-)FCS** | (heat inactivated) fetal calf serum |
| **FITC** | fluorescein isothiocynate |
| **FR(s)** | framework region(s) |
| | |
| **HCL** | hairy cell leukemia |
| **HIS** | histones |
| **HLA** | human leukocyte antigen |
| **HRP** | horseradish peroxidase |

| | |
|---|---|
| **HSP(S)** | heat shock protein(s) |
| **IC** | immune complex |
| **ID** | idiotope/type |
| **IFN-g** | interferon-gamma |
| **(s)Ig** | (surface)immunoglobulin (A, M or G) |
| **IgH** | immunoglobulin heavy chain |
| **IgL** | immunoglobulin light chain |
| **IgV** | variable region of immunoglobulin |
| **IL** | interleukin (-1-10) |
| **IP3** | inositol-triphosphate |
| **J** | joining gene segment of immunoglobulin |
| **kDa** | kilo-Dalton |
| **LCA** | leukocyte common antigen |
| **LDA** | limiting dilution analysis |
| **LMP** | latent membrane protein |
| **LPS** | lipopolysaccharide |
| **MC** | mixed cryoglobulinemia |
| **McAb** | monoclonal antibody |
| **me$^{(v)}$/me$^{(v)}$** | motheaten mice (viable) |
| **MER** | mouse erythrocyte receptor |
| **MFI** | mean fluorescence intensity |
| **MG** | myasthenia gravis |
| **MITO** | mitochondria |
| **MHC** | major histocompatibility complex |
| **MM** | multiple myeloma |
| **MOL. WT.** | molecular weight |
| **MRBC** | mouse red blood cell |
| **muLV** | murine leukemia virus |
| **NC** | nitrocellulose membrane |
| **NHL** | non-Hodgkin's lymphoma |
| **OD** | optical density |
| **PB** | peripheral blood |
| **PBMC** | peripheral blood mononuclear cells |
| **PBS** | phosphate buffered saline |
| **PC** | phosphorylcholine |
| **PEG** | polyethylene glycol |
| **$PIP_2$** | phosphoinositol 4,5, biphosphate |
| **PKC** | protein kinase C |
| **PLC** | phospholipase C |
| **PLL** | prolymphocytic leukemia |

| | |
|---|---|
| **PMA** | phorbol myristate acetate |
| **PtC** | phosphatidylcholine |
| **PTK** | protein tyrosine kinase |
| **PWM** | pokeweed mitogen |
| | |
| **RA** | rheumatoid arthritis |
| **RBC** | red blood cell |
| **RF** | rheumatoid factor |
| **(m)RNA** | (messenger)ribonucleic acid |
| **RT** | room temperature |
| | |
| **SAC** | staphylococcus aureus Cowan I |
| **SCID** | severe combined immunodeficient |
| **(N)SRBC** | (neuraminidase treated) sheep red blood cells |
| **SD** | standard deviation |
| **SDS-PAGE** | sodium dodecyl sulphate polyacrilamide gel electrophoresis |
| **SLE** | systemic lupus erythematosus |
| **SpA** | staphylococcal Protein A |
| **SS** | Sjogren's syndrome |
| | |
| **TCA** | tetra-acetic acid |
| **TdT** | terminal deoxynucleotidyl transferase |
| **TEMED** | tetramethyl-ethylenediamine |
| **TG** | thyroglobulin |
| **TNF** | tumour necrosis factor |
| **TPA** | 12-0-tetradecanoyl-phorbol-13-acatate |
| **TRITC** | tetramethylrhodamine isothiocynate |
| **TWEEN** | polyoxyethylene sorbitan monolaurate |
| | |
| **VIM** | Vimentin |
| **V(H/L)** | variable region gene segment (light /heavy chain) |
| **v/v** | Volume/volume |
| | |
| **WM** | Waldenstrom's macroglobulinemia |
| **w/v** | Weight/volume |
| | |
| **Xid** | X-linked recessive immunodefect |

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# Chapter 1

## GENERAL INTRODUCTION

## Aim and design of the study.

The ultimate aim of the investigations described in this thesis is to expand our understanding of the physiology of the sub-population of human CD5 (B-1) B lymphocytes.

B lymphocytes are a complex population, studies revealing much heterogeneity in their function and definition (Strober et al, 1975, Scher et al, 1982i, Wortis et al, 1982, Mond et al 1982, MacLennan et al, 1982 and Gray et al, 1984).

However, it is reasonable to propose that it has been the development of studies of the murine subset of B lymphocytes, known as CD5 (Ly1), that has raised the most controversy in recent years and has led to careful reconsideration of the basic concept that subsets of B cells are generated from a common bone-marrow precursor (Hayakawa et al, 1985). The point of discussion being, whether this particular subset of B lymphocytes represents a distinct lineage or merely a stage in B cell maturation.

Very recent cell transfer studies favour a separate lineage hypothesis. Evidence was cited, likely defining three murine B cell lineages, conventional B cells, which develop late and are continually replenished from progenitors in adult bone marrow, Ly1+ B cells (B-1a), which develop early and maintain their numbers by self-replenishment and Ly1- B "sister" (B-1b) cells, which share many of the properties of Ly1 but can also develop from progenitors in adult bone marrow (Kantor et al, 1992).

That the presence of a similar set of B cells in such phylogenetically divergent species as mouse and man, suggests that these cells may have an important functional role. Indeed, they have been shown to have regulatory properties (Hara et al,

1988) and helper functions (Sherr et al, 1987).

Although Ly1 B cells constitute a minor subset of B lymphocytes in adult lymphoid tissue (Hayakawa et al, 1983), they have been demonstrated to show clear distinctions in the expression of antibodies, particularly autoantibodies, when compared with "conventional" B cells (Hayakawa et al, 1984). This has focussed much attention on the molecular mechanisms which determine the antibody repertoire of these cells.

A large body of knowledge concerning murine antibody gene organization and diversity has been accumulated (Yancopoulos et al, 1984 and Perlmutter et al, 1985). In comparison, current information concerning the human immunoglobulin gene families is scarce and little is known about the expression of the potential human antibody repertoire.

It is however, becoming increasingly evident that a higher frequency of autoreactive B cells exists in early life in the development of B cells (Dighiero et al, 1985 and Vakil et al 1986), of which CD5 is a major surface molecule (Hardy, 1987).

A greater knowledge of the origin and proportion of CD5 B cells is important for understanding the normal autoantibody immune response.

**Two main issues have been addressed in this thesis :**

1. What is the relationship between CD5 positivity and the frequencies of autoantigen recognizing cells within the human B lymphocyte repertoires of various populations in normal and

disease states ?

2. Is immunoglobulin heavy and light chain variable region gene usage different in human CD5+ compared with human CD5- B lymphocytes?

# 1.1 B CELL DEVELOPMENT

## 1.1.1 DIFFERENTIATION MARKERS DEFINING B CELL DEVELOPMENT

### 1.1.1.1 Organization and rearrangement of immunoglobulin variable (IgV) region genes.

B cell differentiation may be viewed by analysis of immunoglobulin gene expression that follows sequential gene rearrangements (V-D-J and V-J) of the heavy, kappa and lambda loci. This has been extensively studied in the mouse (Alt et al 1986). Since the rearranged V genes expressed by the cell determine its antigen binding specificity, their analysis directly relates to selection at the cellular level.

An immunoglobulin molecule is composed of two types of polypeptide chains, namely the light (L) and heavy (H) chains. Cloning of the mouse kappa ($\kappa$) and lambda ($\lambda$) L chain genes revealed that the variable (V) region is encoded by two gene segments, the variable (V) and the joining (J) genes, coding for the first 95 and last 13 amino acid residues respectively and separated by non-coding nucleotides called introns (Bernard et al, 1978 and Max et al, 1979).

Each V gene segment is preceded by an exon called a leader sequence, coding for a short hydrophobic sequence involved in the transport of the antibody molecule across endoplasmic reticulum membrane during translation. This sequence is removed after synthesis of the chain.

Subsequent cloning and sequencing of mouse H chain genes revealed an extra segment called the diversity (D) segment, encoding variable numbers of amino acid residues (Early et al 1980 and Sakano et al, 1981).

These separate gene segments are brought together and rearranged during B cell development in the primary lymphoid organs.

The genes coding for heavy chains and kappa and lambda light chains were found to be located respectively on chromosomes 12, 6 and 16 in the mouse and 14, 2 and 22 in humans (reviewed by Honjo, 1983).

Studies of Abelson murine leukemia virus (AMLV) transformed mouse pre-B cell lines (Alt et al 1986) and of Epstein-Barr virus (EBV) transformed human pre-B cells (Hui et al, 1989), have revealed that a heavy chain variable region gene is rearranged before a light chain variable region gene. A productive $V_H$- D-$J_H$ rearrangement results in the termination of further $V_H$ assembly (allelic exclusion) and activation of $V_\kappa$ to $J_\kappa$ rearrangement takes place. Light chain isotypes are sequentially rearranged, kappa before lambda. Productive $V_\kappa$-$J_\kappa$ assembly leads to the expression of IgM and the termination of further light chain rearrangement. Exceptions have recently been reported, indicating alternative or concurrent rearrangement of different $V_L$ alleles (Carrol et al, 1988) and of $\kappa$ and $\lambda$ light chain genes (Berinstein et al, 1989).

***a) $V_H$ genes.***

In the $V_H$ locus of man, there are at least 100 $V_H$ genes, 6 functional $J_H$ genes (Walter and Cox, 1988, Kodaira et al, 1986 and Berman et al, 1988) and 30 D gene segments, classified into 6 families (Ichihara et al, 1988).

The $V_H$ gene products have been classified into $V_H$I (34/158, 22%), $V_H$II (16/158, 10%) and $V_H$III (108/158, 68%) based on >75% homology within amino acid sequences of myeloma proteins (Kabat et al, 1987) and the corresponding

genes cloned and sequenced (Walter and Cox, 1988). Further VH families, VHIV, V and VI (Lee et al, 1987, Schroeder et al, 1987 and Berman et al, 1988) have been isolated from DNA libraries and it has been suggested that up to 8 VH families are present in the human VH locus (Newkirk and Capra, 1989).

The human VHIII family appears to be the largest, constituting 40% (Berman et al, 1988) to 50% (Walter and Cox, 1988 and Kodaira et al, 1986) of the total VH genes. In fact this family has now been sub-grouped into VHIIIa and b (Sanz et al, 1989). The VHIV gene family represents the smallest with at least 14 members located most proximal to the D locus (Berman et al, 1988, Schroeder et al, 1988, Pascual and Capra, 1991). In contrast to murine VH genes, the human VH genes are extensively interspersed (Berman et al, 1988).

In the mouse, over 1000 VH genes have been classified into 10 linked and clustered families (Yancopoulos and Alt, 1986). There are in fact striking similarities, at the level of the nucleotide and amino acid sequences between mouse and human VH families. Mouse VH families S107, J606, 7183 and 441-4 are most homologous to human VHIII. The largest mouse family, J558, appears similar to human VHI (Schroeder et al, 1988ii, and Rathburn et al, 1989). Tutter and Riblet, 1989, reported the VHIII gene family to be the most conserved among 20 different mammalian species examined.

***b) VL genes.***

Subsequent to productive rearrangement of the VH genes a Vκ gene segment is recombined to a Jκ gene segment to produce a κ light chain. It has been estimated that there exist about 84 human Vκ (reviewed by Zachau, 1989) and 5 Jκ genes, interspersed within the Vκ locus. There appears to be 4 sub-

groups based on amino acid sequence analysis (Kabat et al, 1987) and gene cloning and sequencing (Klobeck et al, 1987 and Lorenz et al, 1987).

Serological structural differences between members of the VκIII subgroups has allowed further subdivision into VκIIIa and b sub-subgroups (Solomon and McLaughlin, 1971 and Sanz et al, 1989). In the mouse, the Vκ locus is more complex, there being about 100-200 genes classified into 16 gene families (Kofler et al, 1989).

Non-productive rearrangement of Vκ-Jκ genes within both loci results in the activation of lambda light chain gene rearrangement (Korsmeyer et al, 1982). While the number of Vλ genes in the human is not known, 6 clustered Jλ gene segments have been identified (Selsing et al, 1989). It has been suggested that λ light chains can be classified into 7 sub-groups (Chuchana et al, 1990).

However, data from λ light chain transgenic mice argue that there may be a λ lineage of B cells where inhibition of continued L chain rearrangement is not inhibited by a functional κ L chain gene product, as is thought to be the case for most B cells (Gollahon et al, 1988). This may have implications for the previously reported (Hayakawa et al, 1986) levels of λ L chain in Ly1 B cells compared to conventional B cells. Such a mechanism as described above may reflect this observation.

### 1.1.1.2 Regulation and expression of immunoglobulin variable region (IgV) genes.

The kinetics of human Ig gene expression is yet to be clarified although it was reported some considerable time ago that Ig expressing B lymphocytes occurred as early as the 7th week of gestation (Gathings et al, 1977).

During fetal and neonatal development, there appears to be an ordered rearrangement and expression of IgV genes. Studies showed that Abelson murine leukemia virus (AMLV) transformed murine fetal pre- B cells frequently utilize the most D-proximal femily of VH region genes, VH7183 (Yancopoulos et al, 1984). Subsequent studies showed this to be the case during fetal and early neonatal life as well as for pre-B cells (Malynn et al, 1990).

The pattern of VH gene utilization changes 5-7 days postnatal and reaches the adult randomised pattern with a predominant expression of the VHJ558 gene family at 2 weeks of age (Malynn et al, 1990). In man, there exists a predominant rearrangement and expression of VHVI (Schroeder et al, 1987 and 1988) and VHV and VHVI (Cuisinier et al, 1989), the most D-proximal families in man (Schroeder et al, 1988).

However, members of other VH gene families have been found to be expressed in fetal liver (Schroeder et al, 1987). Preferrential rearrangement of Vκ4, 5 and 10 has been reported in murine fetal B cells (Teale and Morris, 1989) and a study of AMLV transformed mouse pre-B cell lines found that the majority rearranged a member of the Vκ4 family (Kalled and Brodeur, 1990) not however the most proximal Vκ gene.

Even so, it may be the physical distance of the V genes (and/or autoantibody related) and the DJ locus that influences

their early expression.

Recent studies of B cell differentiation by analysis of Ig gene expression that follows the sequential gene rearrangement (D-J then V-D-J). Early Ig transcripts from human fetal tissue were identified and it was established the most 3' terminal VHV and VHVI are expressed early (7th week of gestation), in the absence of light chain (Fougereau et al, 1990). By week 11, rapid expansion of the various families leads to the establishment of the adult pattern of expression.

A lambda like germline gene called 14.1, likely to encode a fetal light chain association with the μ chain in pre-B cells, was isolated and it was suggested such a complex would trigger regular light chain rearrangements (Fougereau et al, 1990).

Studies on pre-B cells and transgenic mice suggest that transcriptional enhancers may play a role in regulating gene rearrangement (Alt et al, 1987). Further studies showed that two autoantibody V region genes, hv3005 and VH26, have many short stretches of enhancer sequences and may have potential regulatory elements which influence their expression during B cell ontogeny (Chen et al, 1990i).

Interestingly, the early repertoire of mouse B cells includes a considerable number of clones that rearrange the 7183 VH family and frequently express self-reactivity (Kearney and Vakil, 1986). The most D-proximal VHVI gene family in man also encodes self-reactive antibodies (Logtenberg et al, 1989).

Other members of VHI and VHIII gene families, frequently expressed in fetal liver (Schroeder et al, 1987) also encode autoantibody specificities (reviewed by Chen et al,

1990ii). It seems likely then that regulatory elements such as described above, location of genes and the cellular environment may all influence the rearrangement and expression of V genes.

Furthermore, the early B cell repertoire may be derived from different precursors of B cells from the adult (Jeong and Teale, 1989).

It has been proposed that in primative cells, ie those showing little diversity, gene rearrangement was originally used for and thus selected for, its ability to control the expression of receptor genes, rather than to generate diversity ( reviewed by Janeway, 1992). CD5 B cells could fall into such a category, having been shown to have little or no junctional diversity (Feeney et al, 1990) and appear to arise from a unique early stem cell that is lost as the animal matures (Ikuta et al, 1990, Herzenberg et al, 1989).

### 1.1.1.3 Structure of the variable region of immunoglobulins.

Amino acid sequence studies on myeloma proteins have shown that the variable region of both heavy and light chains is composed of two regions (Wu and Kabat, 1970). These include relatively conserved invariant sequences that contribute to about 85% of the variable region and called frame work regions (FR), separated by highly variable sequences, non-randomly organised and called hypervariable or complementarity determining regions (CDR).

Three CDR's appear to be separated within four FR's. The CDR's are to be found around positions 24-34, 50-56 and 89-97 in the light chains and 31-35, 50-65 and 95-102 in the

heavy chain variable region domains. The variable region of heavy chains has an extra hypervariable region at position 84-91, not thought to contribute to the antigen binding site.

The FR's have been seen to be β-sheet strands connected by 6 flexible loops (Alzari et al, 1988) and it appears that the specificity and affinity of the antigen binding site is ruled by the conformational structure of the 6 CDR's, which is influenced by the interaction of a few residues at specific positions in the CDR and for certain loops in the FR (Clothia et al, 1989).

Humanized monoclonal antibodies, constructed by grafting mouse CDR's onto human FR's, showed that some of the FR residues are crucial for proper folding of the CDR's (Reichmann et al, 1988).

The pattern of amino acids within the FR's allowed their classification into subgroups (Capra and Kehoe, 1975) and sub-subgroups as in the case of VκIIIa,b and VHIIIa,b (Solomon and McLaughlin, 1971, Sanz et al, 1989).

### 1.1.1.4 Cross reactive idiotypes (CRI).

**Idiotype/anti-idiotype** : It is generally accepted that an idiotype (id) represents phenotypic markers of variable region genes encoding the specificity of a lymphocyte antigen receptor.

Each individual antigenic determinent which makes up the idiotype of a given receptor, is termed an idiotope. Idiotypes are historically defined by anti-idiotypic antibodies (αid), raised by immunizing allogeneic animals. Anti-ids. which react only with the immunizing immunoglobulin, define restricted or private ids. Those that are shared between

antibodies define cross-reactive idiotypes (CRI).

Sharing of CRI between antibodies of the same and different specificities, from *different* individuals, may reflect common amino acid sequences within a framework region or complementarity determining region (CDR).

Occassionally, αids define CRI on antibodies throughout many species. This cross-reactivity may not be due to common amino acid sequences within the antibody V regions, but due to the tertiary structure of the αid happening to resemble that of the antigen. Such αids are said to carry the "internal image" of the antigen.

Another type of id has been described which reacts with both the id of the antibody and the antigen recognised by that antibody and has been described as an "epibody" ( reviewed by Bona, 1987 and Chen et al, 1985).

The first evidence of cross-reactivity of ids (Williams et al, 1968), demonstrated the recurrent expression of three different ids. Antisera raised to three different cold agglutinin paraproteins, reacted with 60% of a large panel of cold agglutinin paraproteins collected from different individuals, thereby showing a correlation between the antigen binding site and the expression of ids. CRI were soon accepted to be serological markers for the antigen binding site (reviewed by Kindt and Capra, 1984).

Furthermore, CRI were proved to be inherited (Eichman and Lindt, 1978), implying they may not be restricted to a particular antibody specificity. In fact, CRI are expressed on antibodies with a variety of specificities (Bonilla et al, 1990).

### 1.1.1.5 Major CRI families in rheumatoid factor (RF).

RF's are autoantibodies that bind to the Fc region of IgG antibody molecules (reviewed by Carson, 1981). The idiotypic nature of RF is important.

Using IgM RF paraproteins isolated mainly from patients with essential mixed cryoglobulinaemia (MC) and Waldenström's macroglobulinaemia (WM), certain specific common antigens among monoclonal IgM RF were demonstrated (Franklin and Frangione, 1971). They were classified into two major id families, the Wa (60%) and the Po (20%). The Bla group of CRI was later described (Agnello et al, 1980) which could bind DNA histone and which in fact could be inhibited in an assay by either α-Wa or α-Po CRI.

Polyclonal RF is, however, extremely heterogeneous and either a large number of genes or extensive mutation, contribute to its production (Nelson et al, 1987, Schrohenloher et al, 1990 and Koopman et al, 1990).

*a) Light chain associated CRI* : The vast majority of RF paraproteins sequenced and analysed, have been found to express kappa light chains (Kabat et al, 1987).

Amino acid sequence analysis has shown that the IgM RF from the Wa family are exclusively associated with the VκIII subgroup of light chain (Capra and Kehoe, 1975) and serological analysis has assigned the light chain of the Wa family to the VκIIIb sub-subgroup (Kunkel et al, 1974).

Of the four kappa light chains sequenced so far from the Po family, three belong to the VκIII subgroup and one to the VκI subgroup (Newkirk and Capra et al, 1989).

Two monoclonal αid antibodies have been developed

which react exclusively with a large proportion of either of the VκIIIa and VκIIIb light chains (Crowley et al, 1990). Monoclonal antibodies, 17.109 and 6.B6.6 were raised to VκIIIb (Sie) and VκIIIa (Cor) expressing IgM RF paraproteins.
The reactivity of these antibodies was found to be predominately associated with RF specificity and using these αid antibodies, results have indicated that the CDR2 of the light chains are most likely to contribute to the expression of the Wa molecules (Chen et al, 1987).

The genes coding for these proteins have been characterised using a human VκIII cDNA probe to screen a human placenta DNA library (Radoux et al, 1986 and Chen et al, 1987).

The gene, designated, HumKv325 (kv325), was found to encode the majority of the VκIIIb light chains among the VκIII genes that were cloned and sequenced, thereby demonstrating that the variable region of IgM RF paraproteins can be generated from a single VκIII unmutated, germline gene.

The gene coding for the 6.B6.6 CRI was characterised using a cDNA library from a CLL patient producing 6.B6.6 IgM RF cryoglobulins. These were known to belong to the VκIIIa sub-subgroup (Chen et al 1988). These studies isolated a Vκ gene, designated HumKv328 (kv328). Homology observed between other 6.B6.6 positive light chains and kv328 suggested this gene may encode for the expression of the 6.B6.6 RF paraproteins.

***b) Heavy chain associated CRI*** : Data, including sequencing of members of the Wa family, suggested that the heavy chain may not contribute to the expression of the Wa ids (Chen et al,

1985).

However, monoclonal antibodies, G6 and H1, raised against an IgM RF paraprotein, KOK, from the Wa family, were found to react with the heavy chain only (Mageed et al, 1986i). The G6 CRI is highly associated with RF specificity and exclusively expressed on immunoglobulin molecules from the VHI subgroup (Chen et al, 1985). G6 was also found to be coexpressed with the 17.109 CRI, but not the 6.B6.6 CRI, suggesting that both the heavy and light chains contribute to the expression of the Wa id.

Sequencing of the heavy chain of other members of the Wa family (Newkirk et al, 1987) support the idea of a contribution of the heavy chain to the expression of this id.

Further results (Chen et al, 1989) suggested that the G6 CRI is encoded directly in the germline and in fact the G6 monoclonal antibody is the protein product of the VHI associated germline gene, 51P1 (Schroeder et al, 1987).

Another VHI associated, conformational CRI has been identified by a monoclonal antibody, G8, on a large proportion of the Wa positive RF paraproteins (Mageed et al, 1990).

The B6 and D12 , VH3 associated CRI, were produced using He IgM RF (VHIII/VκIII) as the immunogen (Mageed et al, 1986ii and Crowley et al, 1990). The B6 CRI was found frequently on monoclonal IgM RF molecules and could inhibit the binding of the RF to its IgG antigen (Crowley et al, 1990).

Furthermore, Staphylococcal Protein A (SpA), (see below), could block the interaction of some B6 positive IgM to the anti-CRI. Therefore, B6 CRI is recognised to be a marker for one or a few VHIII genes. The id recognised by the D12

antibody is frequently coexpressed on cells bearing the B6 id in RF paraproteins (R.A. Mageed, unpublished observations).

Autoantibodies in chronic cold agglutinin disease are usually monoclonal and IgM proteins, directed against carbohydrate differentiation antigens, known as I and i (reviewed by Feizi, 1986). A mouse hybridoma antibody reacting with one of the CRI determinants on anti-I and anti-i antibodies and designated R2.1A2 (Silverman et al, 1990), reacts with the VH of proteins from the relatively small VHIV family (Lee et al, 1987). A similar monoclonal called 9G4, raised against an IgM from a patient with lymphoma (Evans et al, 1983) also shows this reactivity.

The antibody called Lc1 (Ono et al, 1987), also reacts with the VHIV family of genes.

Although there is no formal assignment of these CRI to a particular VHIV family gene, recent data indicates an association of these CRI with different germline genes. CRI recognised by Lc1 may be a marker of the 71.2 and 71.4 and related genes (Kodaira et al,1986 and Silverman et al, 1990) and R2.1A2/9G4 a marker for the 4-21 (V58P2) and related genes (Schroeder et al, 1987 and Sanz et al, 1989i).

## 1.1.2. ONTOGENY OF HUMAN B LYMPHOCYTES.

The mature cells of the body arise by differentiation from immature precursor cells through a series of stages, that together, form a lineage.

The differentiation pathway of the B lineage from the lymphoid stem cell to the plasma cell has two main phases, that is, initially the development of resting mature B cells followed by an antigen-dependent differentiation stage. It is generally observed that a characteristic of differentiation is that a high rate of proliferation is coupled inversely to maturation (Mayer et al, 1986).

B cells originate from pluripotent haemopoietic stem cells, generated in the mesenchyme of the embryo. The stem cells migrate to the fetal liver where they differentiate into lymphoid cells (Gathings et al, 1977 and Asma et al, 1984). By the 12th week of gestation, the bone marrow becomes the major and eventually the sole site of haemopoiesis throughout life (Hayward et al, 1981). It has also been recently demonstrated that the human fetal omentum, like the fetal liver and bone marrow, is a primary site of B cell development (Solvanson and Kearney, 1992).

As illustrated in Fig.1.1, the earliest identifiable lymphoid precursors are cells containing the enzyme, Terminal deoxynucleotidyl transferase (TdT) and expressing MHC Class II (Ia) antigens on their surface (Greaves and Janossy, 1978 and McKearn and Rosenberg, 1985). Motheaten (me/me) and viable motheaten ($me^v/me^v$) mice have a defect which, among other abnormalities, leads to the absence of peripheral B lymphocytes. In elegant experiments these mice were shown to be unable to generate TdT and bone marrow lymphoid cells

(Medlock et al, 1987).

Very interestingly, these cells have high numbers of CD5+ B cells and it has recently been proposed that there is a possible role of increasing TdT in increasing receptor diversification (Herzenberg et al, 1989). This is discussed more fully below.

Rearrangement of Ig V region genes, but without the expression of the μ protein, results in self-renewing pro-B cells. These cells rapidly divide to later express cytoplasmic μ H chain and become pre-B cells. They have been detected in the liver at the 8th week of gestation (Gathings et al, 1977). These in turn give rise to small non-dividing cells undergoing Ig L chain gene rearrangement.

The synthesis and assembly of the IgM molecule and finally its expression on the surface of the cell results in immature B cells. Such virgin, IgM positive B cells in the 1° lymphopoeitic organs will co-express IgD (Calvert and Cooper, 1988) by the 13th week of gestation and will migrate to the periphery where they will die or be activated by antigen (Opstellen and Osmond, 1983). The latter situation results in the formation of germinal centres and further differentiation into memory cells or plasma cells (MacLennan et al, 1990).

A small proportion of IgM+IgD+ B cells switch isotype in the human fetus to IgG or IgA. Contrary to adult B cells, the majority of these fetal cells maintain both IgM and IgD on their surface at the same time (Gathings et al, 1977).

One should not lose sight of the fact that much of the information regarding the B cell compartment has been accumulated from studies of established B cell lines and freshly isolated leukemia cells (Nadler et al, 1982, Nilsson and

Totterman, 1984, Foon and Todd, 1986 and 1990 and reviewed by Greaves, 1986). For example, chronic lymphocytic leukemia (CLL), Waldenstrom's macroglobulinemia (WM) and multiple myeloma (MM) represent immature B cells, plasmacytoid and plasma cells respectively. However, consider for example CLL B cells. They express the activation marker, CD23 and the CD5 molecule and therefore do not only reflect any one particular stage.

### 1.1.2.1. B cell surface molecules.

The expression of surface molecules on B cells, as detected by monoclonal antibody reagents, reveals that although most of the antigens are B cell specific, some are not lineage restricted. Some antigens are differentiation associated and restricted in their expression to distinct stages in the B cell ontogeny pathway, while others are associated with activation of the resting B cell.

Furthermore, the particular lymphoid compartment involved and the B cell microenvironment, influence the expression of cell surface antigens (MacLennan et al, 1989, Herzenberg et al, 1987 and Jung, 1986).

In the following and illustrated by Fig.1.1, only the CD cluster antigens (Workshop of Leukocyte Typing : Zola, 1987 and Jung et al, 1986) are discussed. There are, however, several unclustered antigens which may be involved in the regulation of B cell growth and development. For example, perhaps the best known marker is the higher molecular weight epitope of the common leukocyte antigen (designated CD45), known as B220. It is expressed early in the B lineage and continues on mature B cells (Coffman et al, 1982).

More recently a molecule known as BP-1 has been shown to be restricted to the early stages of the B lineage (Cooper et al, 1986). Other determinents such as PB76 (Strasser et al , 1988) and S7 (Hardy et al, 1989) are useful probes to delineate distinct sub-populations of normal early B cells in fetal liver and bone marrow.

i. CD19 is B cell restricted and unrelated to any particular phase of the cell cycle or of B cell differentiation. It is widely used as a pan-B marker. Likewise, CD20, although it is restricted to expression on small, resting B lymphocytes.

ii. CD21 (C3d receptor, CR2 (Iida and Nadler, 1983), is also the receptor for Epstein-Barr virus (EBV) (Fingeroth et al, 1984).

iii. The CD22 molecule is found on mature B cells and interestingly may be involved in adhesion ( Stamenkovic et al, 1991). CD22 is also associated with the receptor for murine erythrocytes (Stamenkovic and Seed, 1990).

iv. CD23 (Blast-2, Fcε receptor) has attracted much interest due to its multiple functions and possible involvement in growth regulation (Gordon et al, 1989). This antigen was originally reported to be an early activation antigen and is highly expressed on EBV transformed B cells (Thorley-Lawson et al, 1985i and ii).

v. CD24 is detectable on cells in the pro/pre B cell compartment (CD24+ sIgM-). It has recently been demonstrated that the density of the CD24 molecule, expressed on bone marrow lymphoid cells, discriminates between pre B and mature B cells (Duperrai et al, 1991).

vi. The CD25 molecule is the α chain receptor for IL-2

vii. CD40 and CD72 are also present on all B cells. Recent

data has shown that the activation of germinal centre cells with anti-CD40 antibody induces the cells to leave cell cycle and acquire the phenotype of small lymphocytes, consistent with the production of memory cells (Liu et al, 1991).

viii. CD44 is a major cell surface molecule, found on most primary leukocytes (Trowbridge et al, 1982). Its functions are not yet clearly defined, but it may play a role in myelo and lymphopoiesis in bone marrow (Miyake et al, 1990). Furthermore, it was recently demonstrated that high levels of CD44 expression distinguish virgin from antigen-primed cells (Camp et al, 1991).

ix Forms of CD45 (leukocyte common antigen) are found on all mature hematopoietic cell types and cell-type specific forms of CD45 are found on distinct cell subsets. These include the B lymphocyte specific from of CD45, B220.

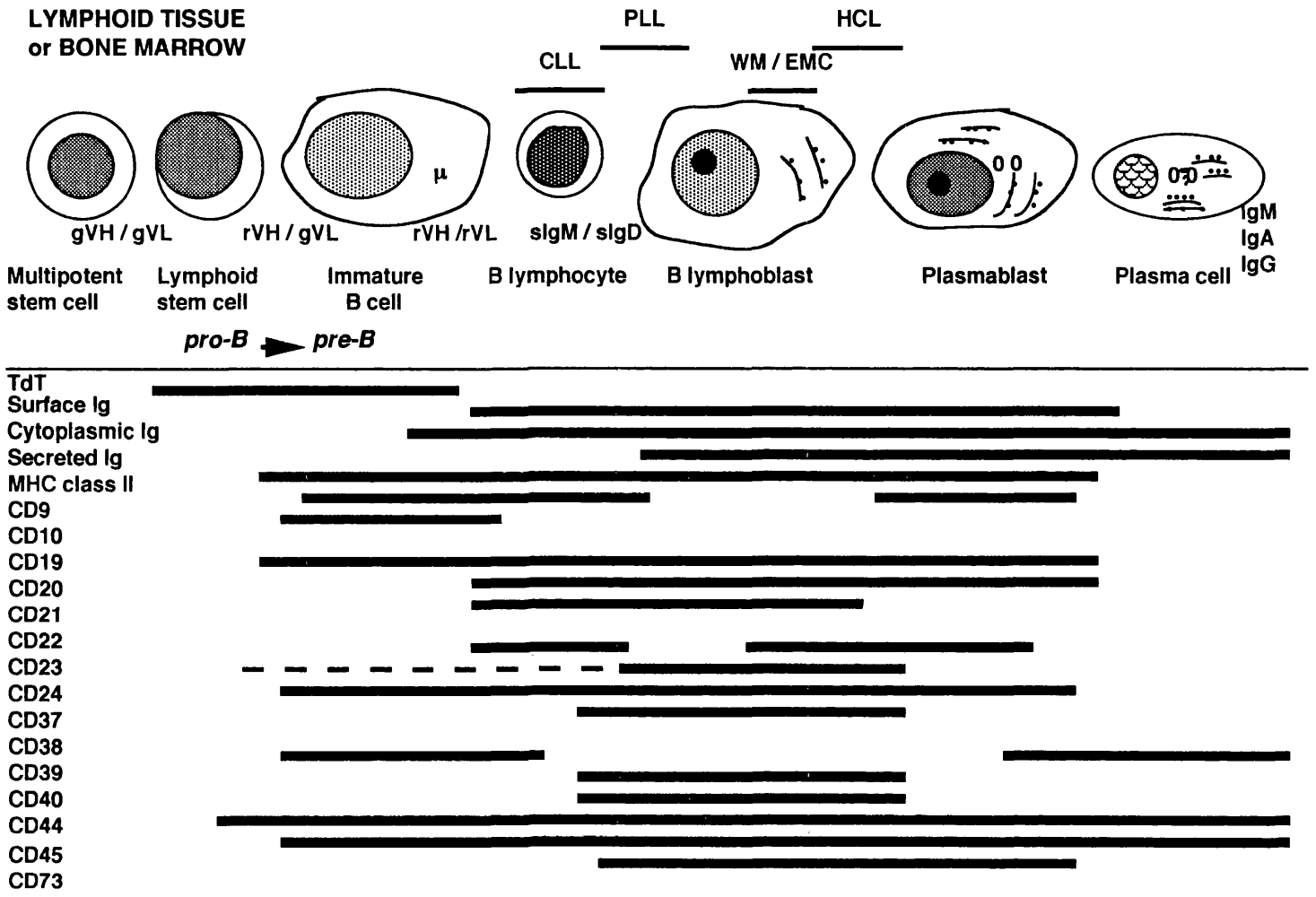


**FIGURE1.1 Schematic model of B cell differentiation and the relation of leukemias/lymphomas ;** *adapted from Nilsson 1978, Greaves 1986 and Zola 1987.*
Abbreviations : CD, cluster of differentiation, HCL, hairy cell leukemia, CLL, chronic lymphocytic leukemia, EMC, essential mixed cryoglobulinaemia, WM, Waldenstrom's macroglobulinaemia

### 1.1.3. ONTOGENY OF CD5 B CELLS.

Murine CD5 B cells differ from conventional B cells in that they appear to not be generated from stem cells present in the bone marrow of adult mice (Hayakawa et al, 1985).

From early cell transfer experiments (Hayakawa et al, 1985 and 1986 and Forster and Rajewsky, 1987), it was concluded that CD5 B cells are generated early in ontogeny and then propagated as mature, surface immunoglobulin positive, B cells for the lifetime of the animal (Herzenberg et al, 1986, Rajewsky et al, 1987 and Hardy and Hayakawa, 1986 and 1988).

CD5 B cells in mice can be detected with the appearance of IgD bearing B lymphocytes in neonatal spleen. In Balb/c mice, CD5 B cells constitute approximately 20% of the surface IgM positive splenic B cells, 5 days after birth (Dexter and Corley, 1987). Similarily, the frequency of peritoneal B cells that coexpress CD5 is high, almost 100%, at 7 days after birth (Hayakawa et al, 1986). Further evidence that CD5 B cells may belong to a separate differentiation pathway comes from the identification of a series of CD5 B cell lines at various stages of differentiation (Davidson et al, 1984). Particular lines were shown to express higher levels of Ly1 with surface IgM. Indeed certain types of culture systems may enrich CD5 precursors. Asialo GM+ cells from newborn liver lack Thy-1 and have been shown to give rise *in vitro* (on a fetal liver derived stromal cell line) to CD5 B cells (Hardy et al, 1987).

In humans, CD5 B cells constitute the major B cell subpopulation in fetal spleen and newborn cord blood (Hardy and Hayakawa, 1986), but by adulthood this subpopulation constitutes <10% of the B cells in normal spleen, lymph nodes and peripheral blood (Gobbi et al, 1983 and Kipps and Vaughan,

1987). This is discussed more fully later.

The self-renewal capacity of CD5 B cells, at least in the mouse, may be correlated with the finding that most B cell chronic lymphocytic leukemias( B-CLL) belong to the Ly1 compartment (Davidson et al, 1984, Hardy et al, 1984 and Haughton et al, 1986). In humans, an equivalent CD5 B cell population has been described (Gadol and Ault, 1986 and Hayakawa and Hardy, 1988), which likewise likely gives rise to the majority of human B-CLL (Boumsell et al, 1980, Royston et al, 1980 and Wang et al, 1980). The CLL B cells are discussed in more detail in a following section.

### 1.1.3.1. CD5 B cells as a discrete B lineage.

There is a considerable body of evidence that would indicate that CD5 (B-1) cells belong to a developmental lineage, distinct from the conventional B lineage.

The recent evidence that characterized the development of three mature murine B cell populations-B-1a, B-1b and conventional B showed that these populations most likely arise from distinct, independent progenitors (Kantor et al, 1992).

Previous experiments have shown that irradiated mice can be reconstituted with CD5 B cells, if the graft contains bone marrow stem cells, together with peritoneal cells (Herzenberg et al, 1986). Further, Severe combined immunodeficient (SCID) mice, lacking in T or B cells, can be immunologically reconstituted with CD5 B cells by injection of fetal liver but not adult bone marrow (Hardy et al, 1986). Very recent experiments with SCID mice have shown that the repopulation of CD5, but not conventional B cells can be

achieved by the injection of fetal cells (Solvanson et al, 1992).

It was previously shown that the CD5 B cell population, developed from pre and neonatal progenitors, survived because they are self-replenishing in the adult. Furthermore, there is a feedback mechanism, through which mature CD5 B cells prevent further CD5 B cell development from their precursors (Lalor et al, 1989).

There appears therefore, to be basic developmental differences between CD5 B and conventional lineage B cells.

The murine peritoneal B cell repertoire is not mirrored in repertoires from either newborn B cells or virgin B cells in adult bone marrow (Lalor and Manohar, 1990). Therefore, either these cells develop from distinct precursors with different mechanisms of variable region gene usage, or newly formed CD5 B cells are heavily selected on specificity, for entry into the peritoneal lineage. If the latter is the case, then, since CD5 B cells in germ-free mice have similar characteristics, then bacterial antigens in the gut, may not be required for such a selection.

Such experiments would seem definitive, but the internal microenvironment in these mice may not be conducive to the development of particular subsets of lymphocytes.

In fig.1.2, a schematic outline of putative conventional and CD5 B cell differentiation pathways are illustrated.

Recently it was proposed that CD5 B cells arise from a fetal stem cell that is originally TdT- and generates CD5+, Ly1+ B cells. As the animal matures, this stem cell is lost, resulting in an adult stem cell, TdT++, which gives rise to a conventional CD5- B cell (reviewed by Janeway et al, 1992).

The role of the products of two pre-B cell specific genes, VpreB and λ5 in B cell differentiation is currently of considerable interest. These form a surrogate light chain that associates non-covalently with the heavy chain, allowing their expression on the surface of pre-B cells (Rolink et al, 1991). It was shown that in the absence of any contacts with stromal cells and/or IL7, surface immunoglobulin bearing (sIg) B cells develop from murine fetal liver cell lines. Furthermore, when injected into SCID mice, the progeny of the cultured pre-B cells repopulate the follicles of the spleen in these mice and are exclusively CD5+.

Although it would appear that murine CD5 (B-1) B cells do represent a lineage, different from classical B cells, it is unclear to which developmental stage these different lineages can be traced back, ie, at which point and by which mechanism they separate from each other. Recent evidence from mouse studies suggested that due to a "developmental switch" in B lymphopoiesis, cell progenitors in early fetal development are committed to a differentiation pathway distinct from that seen in the adult (Hardy and Hayakawa, 1991).

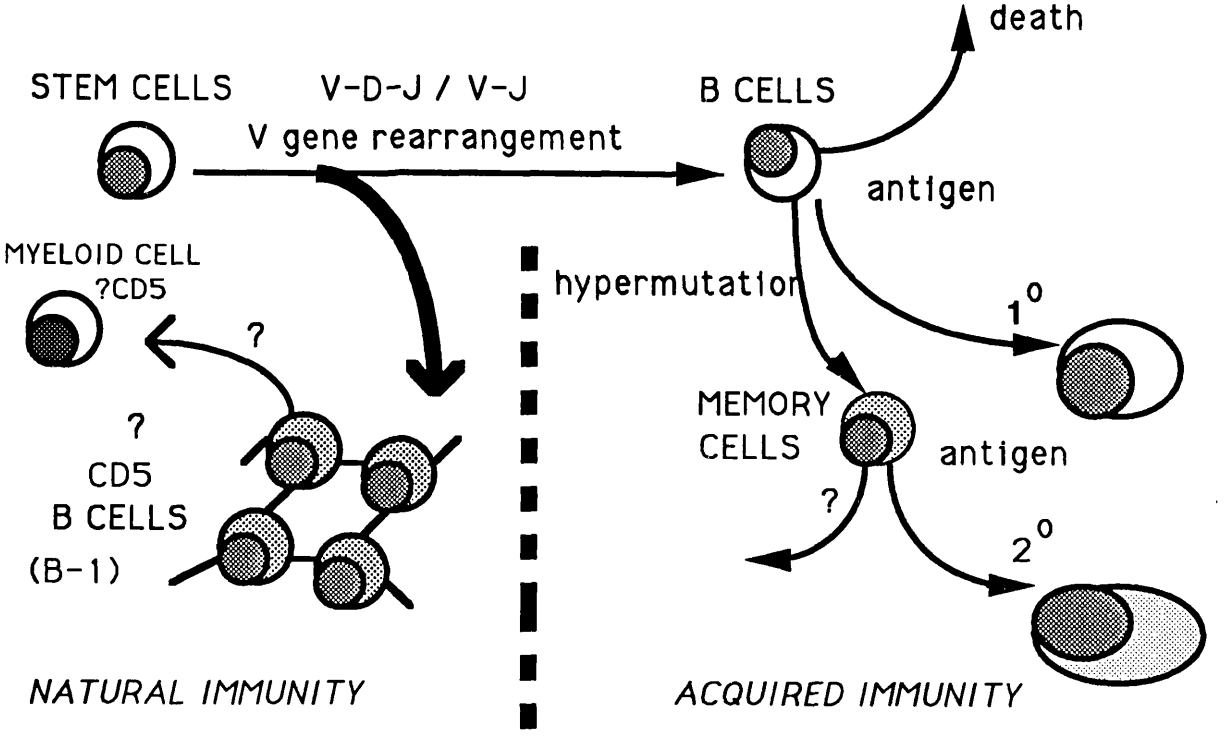


**FIGURE1.2 : Schematic outline of proposed B cell differentiation pathways.**

*Adapted from Rajewsky et al, 1988*

### 1.1.3.2. IgV gene usage by CD5 B cells.

Much of the data on usage of particular immunoglobulin V genes by CD5 B cells has come from studies on lymphoid malignancies.

Spontaneous murine CD5 B cell lymphomas may express immunoglobulin with specificities shared by antibodies produced by non-malignant CD5 B lymphocytes. The CH lymphomas coexpress IgM and CD5 (Pennell et al, 1985) and arose in B10 H-$2^a$ H-$4^b$p/wts mice after adoptive spleen cell transfer and hyperimmunization with sheep erythrocytes (Lanier et al, 1978 and 1982). CRI identified on these lymphomas and nucleic acid sequence analysis of the antibody variable region genes, revealed that several different VH genes were expressed (Pennell et al, 1988). However, there was a high usage of VH11 with Vκ9 and VH12 with Vκ4 (Kantor et al, 1991). Such VH11 usage has been shown to be restricted to CD5 peritoneal cells (Hardy et al, 1989).

Studies in murine autoimmune strains, for example in NZB and related animals, have revealed that these mice, similar to normal murine CD5 B cells, also may express a limited repertoire of IgV genes that have not diversified from germline DNA (Stall et al, 1988i, Tarlinton et al, 1988, Forster et al, 1988 and Reininger et al, 1988). In fact, the mechanism responsible for such expression appears to operate in both normal and autoimmune mouse strains.

It also appears that antibodies produced by human B cell malignancies may share common idiotypic determinents. Using monoclonal antibodies (Carson and Fong, 1983, Mageed et al, 1986i and Posnett et al, 1986), these CRI were found to be expressed by human CLL patients. The products of both the

Humkv325 and 51P1 germline genes were, in fact, coexpressed by a number of the CLL cases (Kipps et al, 1988i). The products of both these genes are also coexpressed by many normal B cells early in ontogeny in the human spleen (Kipps et al, 1990).

Other reports include an overrepresentation of the small VHV and VHVI families of genes in CD5 positive B-CLL cells (Mayer et al, 1990 and Humphries et al, 1988). Furthermore, a high usage of members of the VHIV family of genes has recently been reported (Sanz et al, 1989ii and Schutte et al, 1991).

In conclusion, it would appear that both normal and malignant CD5 B cells, may express different immunoglobulin gene repertoires to that of "classical" CD5- B cells.

## 1.2. CHARACTERISTICS OF CD5 B CELLS.

### 1.2.1. HISTORY OF THE CD5 MOLECULE.

The CD5 surface antigen, originally known as Lyt-1, was first defined nearly twenty years ago as a surface marker for the murine helper-inducer T lymphocyte subpopulation (Cantor and Boyse, 1975). Subsequently, it was shown that in fact the majority of T cells expressed Lyt-1 or Ly1, as it became known (Ledbetter at al 1981).

The human analogue of Ly1, known as Leu1, was then defined as a pan-T antigen (Royston et al, 1980 and Boumsell et al, 1980). However, evidence was produced showing CD5 to be detectable on certain B cell tumours in mice (Lanier et al 1981). Similar data had been obtained from human tumours (Boumsell et al, 1980, Wang et al, 1980 and Royston et al, 1980).

Ultimately, it was soon realised that in both mice and humans, CD5 can be expressed by normal B lymphocytes (Manohar et al, 1982, Hayakawa et al, 1983 and Caligaris-Cappio et al 1982).

The new nomenclature proposed for CD5 B cells, B-1 cells was recently proposed (Kantor et al, 1991). Some investigators find it useful to subdivide the two independently self-replenishing murine B-1 populations into B-1a and B-1b : B-1a cells express CD5 and constitute the majority of B-1 cells in normal animals ; B-1b cells share phenotypic and functional characteristics with B-1a cells, but do not express detectable levels of CD5. It is yet to be resolved if they are to be distinguished and accepted as the new nomenclature.

## 1.2.2. STRUCTURE OF THE CD5 MOLECULE.

Homology between the human CD5 (Leu1) and the mouse CD5 (Ly1) molecule had already been predicted from their distribution on several types of cell (Wang et al, 1980, Lanier et al, 1981 and Ledbetter et al, 1981), when it was confirmed at the DNA level. cDNA clones isolated for both Leu1 (Jones et al, 1986) and Ly1 (Huang et al, 1987), had sequences showing 63% identity along with strong (90%) homology in their carboxyl-terminal regions - illustrated in Fig.1.3.

CD5 has a molecular mass of 67kD and nucleotide sequencing of the CD5 molecule revealed it to be composed of around 470 amino acids (AA) (Huang et al, 1987). The external portion of the molecule contains 22 cysteine residues. There is homology with the immunoglobulin supergene family of molecules on the outer of the two external domains and the inner domain has been shown to be homologous with a chaperone protein, Pap D that mediates assembly of a protein complex on the surface of E. Coli (Holmgren et al, 1989). The transmembrane sequence contains 30 residues and an intracytoplasmic tail of 94AA.

CD72 has recently been identified as a ligand for CD5 on both human and murine B cells (Van der Velde et al, 1991, Van Hoegen et al, 1991).

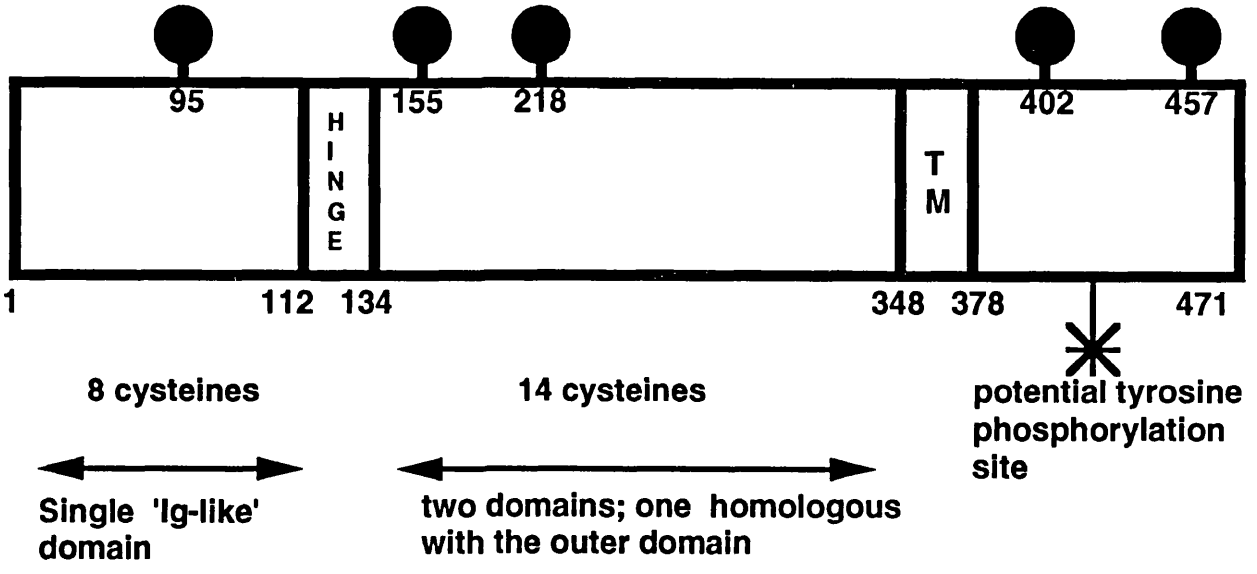


FIGURE 1.3 MODEL OF THE STRUCTURE OF THE CD5 MOLECULE

Potential N - linked glycosylation sites

TM Trans membrane region

Ig immunoglobulin

*adapted from Huang et al PNAS, 84, 204-208, 1987.*

### 1.2.3. SURFACE PHENOTYPE OF CD5 B CELLS.

CD5 B cells are described as lymphocytes that co-express the 67Kd pan-T lymphocyte surface glycoprotein, designated CD5 (Bofill et al , 1985), and surface antigens restricted to the B cell lineage (Hayakawa et al, 1983, Herzenberg et al, 1986, Hardy and Hayakawa, 1986 and Gadol and Ault, 1986).

Most murine CD5 B cells express high density surface IgM and low density surface IgD (Hardy and Hayakawa, 1986). Adult tonsil CD5 B cells in man also have this characteristic (Gadol and Ault, 1988). However, the majority of human CD5 B cells in the blood and spleen of normal adults, express high density surface IgM and IgD and may even express IgG ( Hardy et al, 1986 and Gadol and Ault, 1986).

Like conventional B cells, CD5 B cells express the major B cell antigens, HLA-DR, CD21 (CR2), CDw32, CD19, CD20 (Kipps and Vaughan, 1987 and reviewed by Hardy and Hayakawa, 1986). There is no expression of T cell associated antigens such as CD3, CD4 and CD8. Similarily, murine CD5 B cells coexpress other B cell surface antigens such as ThB (Eckhardt and Herzenberg, 1980) and Ly5 (B22), RA3-6B2, Coffman et al, 1982) and a receptor for Fc of IgG (CDw32) (Dexter and Corley, 1987).

Other surface antigens, associated with activation, such as CD23 and CD25 have been shown to be present on CD5 expressing B cells ( Hardy and Hayakawa, 1986). Furthermore, antibodies to CD5 and CD23 identify two distinct populations in the mouse but not in man (Waldschmidt et al, 1989).

Both murine and human CD5 B lymphocytes have been shown to express CD11b (MAC1, the C3bi receptor) (Beller,

Springer and Schreiber, 1982) and low levels of other monocyte associated antigens such as CD14 (Hogg and Morton, 1987).

Human CD5 B cells have also been shown to bear the mouse red blood cell receptor (MRBC) (Bofill et al, 1985), suggesting they may be overlapping populations (Lydyard et al, 1987). This is supported by the fact that CLL cells of the B cell type are also CD5 positive (Catovsky et al, 1979), and the majority express MRBC receptors normally restricted to a small population of B cells in the fetal lymph nodes and liver. However, phorbol ester (TPA) activated normal B cells will express CD5 (Miller and Gralow, 1984) but not MRBC receptors (Freedman et al, 1987).

## 1.2.4. TISSUE DISTRIBUTION OF CD5 B CELLS.

The CD5 molecule is expressed on the surface of a minor subset of normal adult mouse and human B lymphocytes respectively. In fact, this subpopulation of B cells expresses up to 5 fold fewer CD5 molecules per cell than do T cells (Manohar et al, 1982, Hardy and Hayakawa, 1986, Gadol and Ault, 1986 and Kipps and Vaughan, 1987).

Initially, CD5 was detected, by immunofluorescence and microscopy, in restricted anatomical areas (Antin et al, 1986), but it soon became apparent that these cells constitute a large fraction of B cells early in development (Hardy and Hayakawa, 1986). It followed that the detection and enumeration of CD5 B cells would require sensitive immunofluorescence techniques.

Generally speaking, CD5 B cells constitute 2-3% of normal, adult, peripheral blood lymphocytes and therefore 20-30% of the B cell population (Hardy et al, 1987). They do however, constitute a major B cell subpopulation in fetal spleen (Hardy and Hayakawa, 1986) and newborn cord blood (Bofill et al, 1985 and Antin et al, 1986).

Not detectable in the bone marrow, human CD5 B cells constitute <10% of splenic B cells in most adults (Freedman et al, 1987), but can be detected in lymph nodes and tonsil. Immunohistochemical techniques identify CD5 B cells scattered around the edge of germinal centres (Caligarris-Cappio et al, 1982 and Gobbi et al, 1983). Interestingly, CD5 B cells are frequent during bone marrow regeneration after transplantation (Antin et al, 1987).

Murine CD5 B cells, such as those found generally in the marginal zone of the spleen, are not found in lymph nodes,

blood or bone marrow (Hayakawa et al, 1983). In contrast, CD5 is a major surface antigen on B cells in the murine peritoneal cavity (Hayakawa et al, 1986).

Careful analysis has revealed that the majority of thymic B cells, although themselves a rare sub-population, carry the CD5 molecule (Miyama-Inaba et al, 1988).

### 1.2.4.1. The genetic influence of the expression of the CD5 molecule.

The consistency of the frequency with which CD5 B cells are to be found in an individual's peripheral blood, suggests that their numbers are controlled by genetic as well as environmental factors.

Also, in the mouse, comparison of inbred strains indicate that the level of CD5 expression may be under this type of control. For example, NZB and (NZBxNZW)F1 mice have increased numbers and frequency of CD5 B cells in both the spleen and peritoneum. Most strains have far fewer splenic CD5 B cells (Hayakawa et al, 1983). Likewise, strains such as CBA/N have none detectable (Hardy et al, 1983).

Furthermore, there is great variation in the proportion of CD5 positive peritoneal cells between mouse strains (Hayakawa et al, 1986 and Scribner et al, 1987).

The genetic influence on the expression of the CD5 molecule on B lymphocytes was demonstrated in a study of patients with rheumatoid arthritis (RA) and their healthy relatives. The elevated levels of this sub-population, compared with healthy controls, proved to be a feature of selected RA families rather than distinctive for the proband (Youinou et al, 1990). Indeed, a correlation was detected between the

production of rheumatoid factor (RF) and the percentage of B lymphocytes expressing CD5 in the family groups in the study.

Other evidence lending credence to the possibility of a genetic involvement, is that in contrast to ordinary siblings, monozygotic twins or triplets share similar CD5 levels (Kipps and Vaughan, 1987).

Interestingly, certain ethnic groups, such as the Japanese, are reported to have elevated levels of B lymphocytes bearing the CD5 molecule (Hardy et al, 1986).

### 1.2.4.2. The influence of ageing.

Ageing may affect the levels of CD5 B cells. The proportion of mouse splenic and peritoneal B cells that express CD5, relative to other lymphocyte sub-populations, decreases with age (Hayakawa et al, 1986). This may be accounted for by an increase in the number of B cells that do not coexpress CD5 (Dexter and Corley, 1987).

As already mentioned CD5 cells represent the major B cell sub-population in early development (Hardy and Hayakawa, 1986 and Bofill et al, 1985). Their proportion declines as you move into young adulthood, (Hannet et al, 1992).

CD5 positive malignancies, such as CLL, are common in the older age groups and this could be related to perhaps, an age-related increase in the proportion of B cells positive for CD5. Studies in mice indicate an age-associated increase in the numbers and proportions of CD5 B cells in all tissues, including the peripheral blood (Stall et al, 1988i).

It was recently proposed that the biased use of VH genes, expressed in murine CD5 B cells, may not be the result of a genetic program, but rather a consequence of local, age-

dependent cellular selection mechanisms (Andrade et al, 1991).

## 1.3 FUNCTIONAL CHARACTERISTICS OF CD5 B CELLS.

### 1.3.1 CD5 AS A MARKER FOR B CELL ACTIVATION.

The question of whether CD5 B cells constitute a distinct cell lineage, is difficult to prove conclusively and such direct evidence, ie, isolation or marking of separate progenitors committed to either CD5+ B cells or CD5- B cells, has so far not been presented.

Meanwhile, several investigators, have proposed that B cell expression of CD5 is an indicator of activation of the cells.

#### 1.3.1.1. B cell activation.

The growth and differentiation of resting B lymphocytes are regulated by interactions with soluble factors and by cell-cell contacts. Studies of both human and murine B cells from different lymphoid tissues, have led to the conclusion that the cells need ordered, sequential signals, following on from initial activation to bring the cell out of the Go state and into proliferation and differentiation stages (Howard and Paul, 1983, Kehrl et al, 1984, Kishimoto et al, 1985 and Gordon and Guy, 1987).

Triggering mammalian B cells through their antigen specific receptor leads to transmembrane signals which activate several pathways of signal transduction (second messenger systems). Little is known about the signal transduction within a B lymphocyte, but it appears that at least three different second messenger systems are involved :

i. Firstly, crosslinking of surface immunoglobulin (sIg),

leads to a signal inducing increased phospholipid metabolism (Berridge and Irvine, 1984), with the release of intracellular calcium. It appears that the calcium dependent protein kinase C (PKC) is activated and $Ca^{2+}$ released from its intracellular store (Cambier and Ransom, 1987).

ii. Argueably, the best characterised second messenger system is cyclic (c)AMP (Berridge and Irvine, 1984). Cells carefully regulate the generation and destruction of cAMP, the effects of which are mediated through phosphorylation of its substrates and dependent on protein kinases.

iii. Finally, tyrosine phosphorylation is involved in the signal transduction of many growth factors (Hunter and Cooper, 1985).

Triggering a B cell has been shown to initiate the breakdown of phospholipase c (PLC) to the two second messengers, inositol-triphosphate (IP3) and diacylglycerol (DAG). DAG activates PKC, IP3 initiates the release of intracellular calcium and the combined action of both messengers leads eventually to changes pushing the cell into cycle.

The activation of PKC by phorbol esters and the increase in the $Ca^{2+}$ concentration using calcium ionophores, mimic the effects of a receptor stimulus, suggesting that such signals are sufficient to drive the lymphocytes (Guy et al, 1985).

However, phorbol ester induced differentiation is not generally accompanied by DNA synthesis, (Freedman et al, 1987) and studies have indicated that depending on the type of initial activation signal and which co-stimulatory factors are used, B cells have the option to both proliferate and

differentiate, or just differentiate (Melchers and Anderson, 1984 and Kishimoto et al, 1985).

Crosslinking of either immunoglobulin or Class II molecules on the surface of the B cells, in addition to activating PLC, leads to the appearance of several tyrosine phosphorylated proteins. This suggests that protein tyrosine kinases (PTK), are activated when antigen specific receptors are cross-linked (Lane et al, 1990). In fact, recent studies have indicated that the engagement of multiple B cell surface molecules initiates a signal transduction cascade that involves tyrosine kinases, but not PKC, and which leads to adhesion mediated by distinct cell surface adhesion receptors (Kansas and Tedder, 1991).

It has been proposed that signal transduction in B cells proceeds by the activation of PTK which catalyses the activation of PLC, with calcium providing a central control point. The fact that the activation of PKC by phorbol ester, PKA by cAMP and the tyrosine phosphatase, CD45, by monoclonal antibody, all inhibit the calcium signal, suggests these proteins may influence the downstream activation processes that take place after the activation of PTK.

In human and murine B cells, the intracytoplasmic portions of IgM and IgD contain only 3 amino acids, which suggests a role for any associated proteins able to translate the receptor signals (Cambier et al, 1987). It now seems that in B cells, similar to the CD3 complex on T cells, surface immunoglobulin receptors are associated with a protein complex. IgM is associated with a 34kD protein (Hombach et al, 1988 and 1990), encoded by the mb-1 gene (Sakaguchi et al, 1988) and IgD is associated with similar but distinct proteins

(Wienands et al, 1990 and Campbell et al, 1990). It is yet to be explained how these proteins are linked to the activation of PLC. Indeed, accessory proteins are likely to be necessary for signal transduction (Justement et al, 1990).

Once stimulated the resting (Go) B cells increase the rate of RNA synthesis. An increase in cell size is obvious and ensuing expression of cell surface antigens may include those that are receptors for growth factors (Kehrl et al, 1984).

### 1.3.1.2. Effects of activators and cytokines on B cell CD5 expression.

The suggestion that the expression of the CD5 antigen is an indicator of B cell activation was supported by studies demonstrating that the phorbol ester, phorbol myristate acetate (PMA) can induce malignant and normal CD5- human B lymphocytes to express CD5 (Miller and Gralow, 1984) and B cell antigens such as CD25 (Waldmann et al, 1984) and CD23 (Thorley-Lawson et al, 1985i).

Such an observation cannot be explained by the outgrowth of a sub-population of CD5 B cells within the culture system (Hardy and Hayakawa, 1986, Youinou et al, 1987 and Freedman et al, 1987).

Murine B lymphocytes appear to be unresponsive in that their expression of the CD5 antigen is not affected by mitogen treatment. Neither lipopolysaccharide (LPS) (Hayakawa et al, 1984) nor phorbol esters (Hardy and Hayakawa, 1986) induce these cells to express CD5. In fact, activation by LPS down-regulates the Ly1 antigen (reviewed by Hayakawa, 1990).

Similarily, IL4 down-regulates the CD5 surface

expression on human normal and malignant B cells (DeFrance et al, 1989).

However, treatment of murine CD5- B cells with anti-Ig, but not LPS, induces surface CD5 and increased CD44 (Cong et al, 1991). This data suggested that conventional CD5- B cells can be activated by either of two pathways; one generating CD5+ B cells, the other yielding conventional activated B cells. It was hypothesised that sIg cross-linking corresponds to T independent (type 2) stimulation, while cognate interaction with helper T cells, in the absence of sIg cross-linking, induces B cells to enter the second pathway.

Interestingly, oestrogen does not affect the number of CD5 B cells (Ansar et al, 1989). This sex hormone is known to accelerate experimental autoimmune disease and may be an activator of the CD5 subset (Ansar et al, 1985). By measuring autoantibodies to bromelein treated mouse erythrocytes (Br-MRBC) and quantitating lymphocytes using flow cytometry, an increase in the number of antibody forming cells was demonstrated. This resembles the effects of LPS which increases the antibody forming cells to Br-MRBC but not the expression of CD5 (Herzenberg et al, 1986).

Other data has shown that adult blood CD5- cells may transiently express CD5 following activation by mutagenised EL4 cells and T cell factors (Werner-Favre et al, 1989).

There were initially indications that the CD5 surface molecule may serve as a cytokine receptor. Most notable were the findings that IL1 and anti-CD5 monoclonal antibodies have similar activities *in vitro* (Ceuppens and Baroja, 1986). However, both members have now been cloned and although share characteristics of the same immunoglobulin supergene

family, they are distinct (reviewed by Kipps, 1989).

Interesting experiments, displayed that there was an overactive response by CD5 B cells in the autoimmune prone NZB x NZW(B/W) F1 young mice to IL2 (Hasegawa et al, 1989). Unlike the parental NZB and NZW, the B/W F1 mice showed a rapid decrease in the proportion of splenic CD5 B cells at around 6 months of age.

CD5 B cells from NZB/W mice, display this type of hyper-response to IL5 *in vitro* (Umland et al, 1989) and also produce autoantibodies in response to this cytokine (Herron et al, 1988). Furthermore, an expansion of the CD5 B cell population has been observed in mice transfected with the IL5 gene, suggesting, that the CD5 B cell, may be more sensitive to IL5 induced proliferation, than conventional B cells (Tominaga et al, 1991).

However, other autoimmune strains failed to display this characteristic. IL5 could of course be contributing to the disease in these animals.

Perhaps the *in vitro* cellular environment should be taken into consideration. Recently, an IL3 dependent progenitor B cell line was shown to differentiate into CD5+ and CD5- progeny, depending on the culture conditions (Palacios et al, 1989).

Some interesting experiments have employed a murine CD5+ hybridoma factor to induce the expression of CD5 on CD5- B cells (Gibson, Hardin and Sherr, 1990) and others have shown that IL10 is produced by CD5+ peritoneal although not by splenic B cells (O'Garra et al, 1990). In fact, anti-IL10 introduced *in vivo* prevents the development of all murine B cells in the peritoneal cavity (O'Garra, unpublished). This

would suggest that IL10 may be an autocrine factor for the production of B cells at that site. Furthermore, CD5 B cells have been shown to inhibit T cell functions (Farkas et al, 1987). IL10, which is also produced by TH2 cells, is known to inhibit the production of cytokines by THI cells (Mossman et al, 1990), probably through antigen presenting cells (Fiorentino et al, 1991)

CD5 B cells may respond differently to exogenous stimulators simply because they have a different susceptibility and one cannot conclude that they are merely a sub-population of activated normal cells. Studies of Balb/c or NZB lymphocyte populations, enriched for CD5 B cells have indicated that they are normally in the resting Go or G1 phases of the cell cycle (Forster and Rajewsky, 1987) and most likely are not activated B lymphocytes.

CLL B cells express the CD5 molecules (Whiteside et al, 1977, Gordon et al, 1983 and Freedman et al, 1987ii) and it has been proposed that the phenotype of B-CLL corresponds to that of activated, predominantly immature B cells. Although phorbol ester stimulates the expression of CD5 together with other activation antigens (Freedman et al, 1987ii and reviewed by Freedman and Nadler, 1988), these cells undergo differentiation without proliferation (Forsbeck et al, 1987 and Larsson et al, 1987). Furthermore, during Go to G1 transitions, the cells alter the expression of several surface antigens and c-myc RNA in a manner resembling that observed for normal B lymphocytes once activated (Clark and Ledbetter, 1986).

It has been proposed that murine CD5 (B-1) cells could derive from progenitors that require contact with antigen in a particular form (Kantor, 1991). Evidence of this comes from

experiments, in which, treatment of conventional B cells (CD5-), with anti-immunoglobulin antibodies and IL6, leads to a cell surface phenotype that closely resembles that of the B-1 cells (Ying-zi et al, 1991).

## 1.3.2. ANALYSIS OF THE HUMAN CD5 ANTIBODY REPERTOIRE.

### 1.3.2.1. Epstein-Barr virus (EBV) immortalization

The use of a polyclonal B cell activator is needed to express the antibody producing potential of resting B lymphocytes, without involving T cells (Waldmann et al, 1982).

The murine system allows the use of a number of such activators, for example, dextran sulphate or bacterial lipopolysaccharide (Waldmann et al, 1982). In the case of human lymphocytes, the only efficient T cell independent B cell activator is Epstein-Barr virus (EBV). EBV has become a powerful tool to examine the diversity of the human B lymphocyte repertoire and has allowed the quantitation and characterisation of B cells committed to the production of different types of antibodies.

EBV is a human herpes virus which preferentially infects the cells of the B lineage (Jandau et al, 1973). It drives B cells to differentiate into immunoglobulin secreting lymphoblasts that express surface markers related to activated B cells (Kinter et al, 1981 and Thorley-Lawson et al, 1985i and ii). Many of these infected cells have unlimited growth potential *in vitro* and in fact, the majority of studies using the EBV system have concentrated on this ability of the virus to induce proliferation into permanent lymphoblastoid lines.

The EBV gains entry to the B cell through specific binding to the C3d receptor molecule (CR2, complement receptor type II, CD21) on the cell surface (Fingeroth et al, 1984). This is sufficient to send a minimal signal to the B cell

(Gordon et al, 1986), but full activation followed by establishment of an "immortal" cell line, requires the expression of a subset of viral genes encoding a series of nuclear antigens (EBNA1,2,3 and LP) and a latent membrane protein (LMP) (Dambaugh et al, 1986). Immediately after infection and before virus induced DNA synthesis, the cells express the activated antigens, Blast1 (CD23) and 2 (Thorley-Lawson et al, 1985i). Production of leukocyte migration inhibition/leukemia inhibitory factor (LIF), early in the activation process (Masucci et al, 1984), precedes proliferation (reviewed by Roklin, 1980). Interestingly, LIF is a cytokine with a broad range of activities, that in many cases, parallel those of interleukin 6 (IL6) (Gearing et al, 1991).

The B cell surface phenotype changes dramatically and activation antigens such as the 45kD protein, CD23 and the 80kD protein, CD39 (Kinter et al, 1981 and Rowe et al, 1982) are expressed. The expression of EBNA 2 selectively upregulates the expression of CD23 (Wang et al, 1987). Most interesting will be the establishment of the identity of the target B cell for transformation in the EBV system.

Although most human B cells express receptors for EBV, few (usually <1%) are readily transformed (Steel et al, 1987 and Yarchoan et al, 1983). It was reported in recent studies (Crain et al, 1989) using tonsillar B cells, sorted on the basis of their expression of the early activation antigen, Bac-1 (Suzuki et al, 1986), that EBV responsiveness was dependent on an early activation signal. Bac-1+ tonsil cells were found to be deficient in their expression of surface IgM and IgD, ie, these unresponsive cells may be more differentiated and may have undergone isotype switching.

This is consistent with the observations that the frequency of EBV transformed cells committed to IgM secretion is much greater than that of other isotypes (Tosato et al, 1980). Also there is a lower rate of EBNA expression in high density IgG+ cells compared with IgM+ or IgD+ (Aman et al, 1985). Such cells may also require a priming event to allow transformation to proceed.

EBV transformed B cells generally remain proliferating in culture but do not fully differentiate. Neither is there any detectable isotype switching (Yarchoan et al, 1983).

In the murine system, viral transformation using Abelson leukemia virus has led to the immortalization of B cells at various stages of B cell development (Alt et al, 1986) and similarily in humans, certain pre-B cells, lacking detectable surface immunoglobulin, have been shown to be transformable *in vitro* using EBV (Fu et al, 1984, Katamine at al, 1984).

The establishment of cell lines from chronic B cell disorders has always proved difficult. Except for Burkitt's lymphoma cells (Nilsson et al, 1979), neoplastic B cells, including CLL CD5+ B cells, have proved to be a problem to maintain. This contrasts with the ease with which normal B cells can be induced to grow continuously by means of *in vitro* infection with EBV ( Klein and Klein, 1984).

### 1.3.2.2. Antibodies secreted by EBV infected cells.

It was demonstrated that 35% of human B lymphocytes immortalized by EBV, secrete immunoglobulin with autoantibody activity (Avrameas et al, 1988).

Several groups have now isolated and examined the normal, human, adult CD5 B cell repertoire, by transforming B lymphocytes with EBV, followed by limiting dilution analysis ( reviewed by Casali and Notkins, 1989).

They found that the CD5 B cell population is responsible for secreting autoantibodies of the IgM type. The majority of these bind with low affinity and are polyreactive.

A selected expansion of clones of B cells has not been discounted, however increasing reports suggest that this is probably not the case. Very recent data has demonstrated that EBV transformed neonatal blood B cells exhibit immunoglobulin gene usage, similar to untransformed populations (Guigou et al, 1991).

## 1.3.3. CD5 B CELLS IN HEALTH.

### 1.3.3.1. *Natural* autoimmunity.

Autoreactivity is the recognition of components of an individual by antigen receptors of that individual's immune system.

Recognition of self-antigens of the MHC (Katz et al, 1973) and recognition of antigen binding receptors in an idiotypic regulation of the immune response are basic processes (Jerne, 1974).

Clearly, numerous studies have established that healthy individuals have B lymphocytes capable of recognizing autoantigens (Rose and Witebsky 1956, Sawada et al, 1979, Guilbert et al, 1982, Avrameas et al, 1981, Holmberg et al, 1984, Digheiro et al, 1982, 1983, 1985 and 1986, Koopman et al, 1986, Fish et al, 1984 and Cairns et al, 1985) and that such autoreactive B cells rather than being "forbidden" clones (Burnet, 1959), appear to be quite normal.

**a) Neonatal immune response.**

The immune response in neonates differs from those in adults, likely a result of the immunocompetence of these young cells.

A major contributing factor to this situation in the fetal environment must be the subpopulations of cells present. Indeed, the majority of the B cells, as has been discussed, express the CD5 molecule (Hayakawa et al, 1986).

In both neonatal mouse and man, IgM is the principal serum immunoglobulin isotype (Gathings et al, 1977), in contrast to adults, where IgG predominates. However, although

IgM is synthesised in the human fetus, serum concentrations in the newborn are much less than the adult levels.

This may reflect a lack of antigenic stimulation *in utero.* Human infants are known to have a poor capacity to mount responses to particular antigens, for example, T cell independent carbohydrate antigens, resulting in rendering them susceptible to bacterial infections such as Streptococci pneumoniae (Gathings et al, 1977).

The concentration of non-maternal IgG is very low in the newborn and indeed reponses to most antigens are restricted to IgM antibodies. This may, at least in part, lie with the T cells, as data has shown that neonatal T cells are poor promoters of IgG production in mitogen stimulated cultures of adult cells (Tosato et al, 1980). It was demonstrated that cord blood lymphocytes failed to produce immunoglobulin in reponse to a T cell dependent polyclonal activator such as PWM, but did respond to a T cell independent one such as EBV (Tosato et al, 1980).

Therefore both B and T cells in human newborns have signficant functional differences when compared with adults. Interestingly, although B cell activation into immunoglobulin secreting cells is deficient in early cells, at birth the percentages of peripheral blood B cells bearing surface IgM and IgA is within the range found in the adult (Gathings et al, 1977). This lag between the emergence of B cells and their ability to secrete antibody is curious and is somewhat comparable to the lymphocytes involved in the disease hypogammaglobulinemia.

It has also been shown that the fetal repertoire of antibody specificities is restricted (reviewed by Klinman and

Linton, 1988). For example, as previously described, B cells in Balb/c mice bearing the T15 idiotype and responsible for responses to phosphorylcholine (pneumococci polysaccharide) are delayed in appearance until 7 days after birth. This is similar to B cells responsible for responses to the carbohydrate antigen, β2-1 fructosan.

This is likely to be related to the suggested biased recombination and expression of the IgV region gene families (Alt et al, 1987), as discussed earlier.

**b) Natural antibodies.**

***i. Human***

Studies have shown that in normal human serum, natural antibodies exist that are able to react with internal and membrane bound cell components as well as with circulating proteins, polysaccharides and nucleic acids (Guilbert et al, 1982).

The appearance of anti-DNA antibodies in normal individuals has been well reported (Rubin and Carr, 1979, Hoch et al, 1983, Clough et al, 1980 and Cairns et al, 1984 and 1985). The majority of natural antibodies react with more than one antigen and almost always recognise self-components.

Interestingly, human monoclonal immunoglobulins derived from patients with immunoproliferative diseases, have specificities similar to these natural antibodies and therefore may represent an expansion of a clone of these B cells (Dighiero et al, 1983). Although IgM antibodies predominate, IgA and IgG have been observed (Seigneurin et al, 1988).

Analysis of antibodies secreted by human EBV infected B cells or from human-mouse heterohybrids, have

shown a similar pattern of multispecificity by binding to Fc of IgG, ssDNA, thyroglobulin and insulin (reviewed by Avrameas, 1988).

*ii. Murine*

Antibodies with multiple specificities and autoreactivity have been found in the sera of normal mice (Dighiero et al, 1983). Furthermore, natural antibodies found in adult mice correspond to antibodies raised after infection of mice to environmental pathogenic agents, that cross-react with self-antigens (Karray et al, 1986). B cells synthesising natural antibodies are also present in newborn, nude and germ/antigen free mice and often in higher numbers than in the adults (Dighiero et al, 1985 and Pereira et al, 1986).

*iii. Possible functions of natural immunoglobulins*

A number of proposals have been put forward as to the role of natural antibodies.

Several years ago, Grabar proposed that natural antibodies, by binding to catabolic and metabolic substances, contribute to their clearance. More recently, it has been suggested that, B cells carrying natural polyspecific antibodies as receptors after stimulation by an antigen, would be induced to undergo a series of divisions and mutations, which would lead to the production of highly specific antibodies for a given epitope of that antigen (Avrameas et al, 1983, Dighiero et al, 1983). Indeed, natural immunoglobulins may be able to react with pathogenic agents such as bacteria and parasites, and to enhance their opsonization (Cross et al, 1989, Navin et al, 1989). Furthermore, natural immunoglobulins may also enhance the opsonization of damaged self-constituents (Lutz et al, 1987, Hinter et al,

1987), and have been known to contribute to resistance against tumours (Chow and Bennet, 1989).

Natural antibodies may function as a filter, preventing autoantigens from inducing a powerful immune response that may be triggered by cross-reacting antigens on infectious agents (Cohen and Cooke, 1986) or they may enhance a response induced by antibodies formed against a foreign antigen, by forming a complex with self antigens, similar to the role of MHC (Shoenfeld et al, 1989).

### 1.3.3.2 CD5 B cells are *naturally* autoreactive

#### a) Features of antibodies secreted by CD5 B cells

In humans, it was first indicated that CD5 cells secrete autoantibodies when it was observed that B cells bearing the MRBC receptor were enriched for cells that synthesise RF after stimulation with EBV (Fong et al, 1983).

Much later, sorted normal, adult B lymphocytes that were CD5+, were transformed and shown to produce IgM antibodies with affinity for RF and ssDNA (Casali et al, 1987).

Further studies on CD5 B cells stimulated with Staphylococcal aureus Cowan I (SAC), produced large amounts of RF compared with "conventional " B cells (Hardy et al, 1987).

Also, in man, fusion of EBV transformed cells from CD5 B cells, with mouse heterohybrid cells resulted in monoclonal antibodies which bound to the Fc of IgG and other self-antigens such as ssDNA, thyroglobulin and insulin (Nakamura et al, 1988). Indeed, a high proportion of these antibodies were multispecific. Similarily, in the normal mouse, both the splenic (Hayakawa et al, 1984) and peritoneal

(Pennell et al, 1989), CD5 B cell subset has been shown to express autoantibodies to PtC.

**b) Possible function of CD5 antibodies in health**

It is likely that CD5 B cells contribute to the *natural* autoantibody secretion in serum (Casali and Notkins, 1989), such as that described above.

As a first line of defence, such antibodies would be invaluable against invading microorganisms until a specific antibody response could take place.

It has been suggested that immunoglobulins produced by these cells are directed against the polar head groups of phospholipids (Mercolino et al, 1988, Cox and Hardy, 1985 and Kawaguchi, 1987) which form the basic matrix of all cell membranes.

Further, it has been since proposed (Van Rooijen, 1989) that bacterial endotoxins trigger the production of these autoimmune and phospholipid antibodies by CD5 B cells. Cross-linking of their immunoglobulin receptors by such antigen would lead to antibody secretion.

### 1.3.3.3. CD5 B cells and an immune network

It would seem then, that CD5 B cell derived antibodies would allow the recognition of self and non-self external antigens.

Such a system would enable the body to recognize and respond to structures through receptors that are polyspecific.

This could be regulated, as proposed by Jerne, through an active network system where receptors (anti-idiotopes) would interact with complementary proteins (idiotopes) and

establish relationships, which would in turn lead to a homeostasis.

CD5 B lymphocytes activated by antigens in the internal environment may then express an exclusive repertoire. This repertoire may use a network of highly connected antibodies which would control the activated cells.

Evidence from the activation of B cells in normal and germ-free mice supports this (Pereira et al, 1986 and Holmberg et al, 1984). Therefore activation of autoreactive cells may be a primary event in the control of the immune system.

B cells early in their development (and generally CD5 positive), produce autoantibodies in the mouse directed to highly connected immunoglobulin idiotopes, encoded by germ-line V genes (Vakil and Kearney, 1986 and Holmberg et al, 1987). That these anti-idiotypic antibodies are of significance was shown by injecting them into neonates. Consequently, the development of idiotype bearing antibodies to certain antigens in the adult animal was modified.

In this regard, a human CD5 B cell clone has been produced which secretes a high affinity anti-idiotypic antibody which is suggested as being involved in the maintenance of immunological memory (Van de Heijden et al, 1991).

## 1.4. CD5 B CELLS IN DISEASE STATES.

For some time, evidence has been accumulating to suggest that the mechanisms controlling non-organ specific autoimmune diseases, for example, polyclonal B cell activation, are similar to those leading to lymphoproliferative disorders (Seligmann et al, 1984).

Since a limited number of clones are likely to be involved in these diseases, it is possible that the CD5 B cells may be responsible for autoantibody production in the first type of disorder and for susceptibility to malignant transformation in the latter.

The relationship between B cells expressing CD5 and disease is therefore of some interest and an understanding of the regulation and function of this subpopulation of B lymphocytes may shed some light on the pathogenic mechanisms operating in these disease states.

### 1.4.1. FEATURES OF CD5 IN B CELL MALIGNANCY

#### 1.4.1.1. Human leukemia and lymphoma.

Chronic lymphocytic leukemia (CLL), was first reported by Benneth (1845), but it wasn't until fifty years later that the unique features of CLL as a distinct leukemia were described (Turk et al, 1903).

CLL is the most common leukemia of adults in western countries, but less frequent in asia (Gale and Foon, 1985). In 95% of the cases, CLL represents an expansion of a single clone of neoplastic B cells.

Three separate entities have emerged, CLL, prolymphocytic leukemia (PLL) and hairy cell leukemia (HCL), as illustrated in Fig.1.1 The probability of acquiring B-CLL

increases with age and is twice as frequent in males.

An association between lymphoproliferation and autoimmune disease is documented and immunoglobulins derived from B cell proliferations may be autoreactive (Hamblin et al, 1986). It has been proposed that a common genetic factor predisposes for the development of the malignancy (Conley et al, 1980). B-CLL clones have been described as mature, resting B lymphocytes or weakly preactivated (Fu et al, 1974 and Salmon and Seligmann, 1974.)

It has also been reported that surface IgM is expressed at a lower density on CLL B cells and often simultaneously with IgD (Gale and Foon, 1985). The majority of the B-CLL clones express MRBC receptors (Catovsky et al, 1979 and 95% coexpress the CD5 antigen ( Freedman et al, 1987 and Pluta et al, 1990.

Leukemic B cells in not all cases of PLL express the CD5 molecule (Den Ottolander et al, 1985, Berrebi et al, 1990 and Gobbi et al, 1983) and malignant cells forming HCL have only been occasionally reported to express CD5 (Caligaris-Cappio et al, 1984 and Den Ottolander et al, 1985). Similarily, the CD5 trait is not shared by immature B cell malignancies such as pre-acute lymphoid leukemia or end-stage differentiated B cell malignancy. For example, multiple myeloma (MM) or Waldenström's macroglobulinaemia (WM) (Goggi et al, 1983 and San Miguel et al, 1986).

However, more recently, B cells that secrete monoclonal IgM anti-myelin associated glycoprotein in a patient with IgM monoclonal gammopathy, have been shown to belong to the CD5 subset (Lee et al, 1991). Less than half of the B cell derived non-Hodgkins lymphomas (NHL) are CD5 positive (Borowitz et al, 1985). In fact, it was observed that

malignant cells expressed CD5 more frequently when the tumours were associated with a peripheral blood lymphocytosis (Medeiros et al, 1987).

The phenotype of these CD5 B cell lymphomas or leukemias cell is reminiscent of the lymphocytes to be found in normal primary follicles and the mantle zone of secondary follicles (Caligaris-Cappio and Janossy, 1985). It was proposed (Salmon and Seligmann, 1974) that the phenotype of B-CLL corresponds to that of normal B cells arrested at a stage of differentiation between pre-B and mature B cells. As mentioned in an earlier section, reports indicated that B-CLL in fact corresponds to a subpopulation of activated cells (Gordon et al, 1984 and Freedman et al, 1987ii), based on data showing that TPA stimulated spleen cells express CD5 and activation antigens. Contrary to this, anti-Ig stimulated normal B cells do not express CD5 and TPA activated normal B cells do not express MRBC receptors. Thus, it would seem that perhaps B-CLL clones represent cells arrested at slightly different stages of B cell development.

The proliferative capacity of CD5+ B cells is greater than their CD5- counterpart and cultures of peripheral blood lymphocytes show that CD5+ cells survive longer,and it has been suggested that the proliferating clones in CLL could originate from the CD5 expressing B cell subset.

### 1.6.1.2. Murine malignancy.

Several murine B cell lymphomas that have developed in ageing mice have been reported to coexpress CD5. For example, the BCL1 leukemia that occurred spontaneously in an ageing Balb.c mouse (Slavin and Strober, 1978).

The CH series of lymphomas arose in ageing recipients of adoptively transferred syngeneic spleen cells after hyperimmunization with sheep red blood cells (Lanier et al, 1978,1982). These coexpress CD5 with surface IgM (Pennell et al, 1985). Similarily, a lymphoma expressing CD5 has been described in ageing NFS/Nr, virally induced, oncogenic mice (Davidson et al, 1984).

## 1.4.2. CD5 B CELLS AND AUTOIMMUNE DISEASE.

### 1.4.2.1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune condition affecting up to 3% of the population and has a female preponderance.

Although various organs can be involved, the disease primarily affects the joints and results in inflammation and damage, persisting for many years and causing deformity and a poor quality of life. There is a frequent association with HLA-DW4 and HLA-DR4, suggesting a genetic factor is involved (Lotz and Vaughan, 1988, Harris, 1989).

Furthermore, certain microrganisms, including EBV, parvovirus and mycobacteria, have been suggested as triggers (Alspaugh et al, 1981, Keat et al, 1986, Phillips et al, 1986, Reid et al, 1985 and Stollerman et al, 1989, Tsoulfa et al, 1989).

Drug therapy is used to treat RA, initially non-steroidal, anti-inflammatory agents, followed by drugs such as D-Penicillamine, Azathioprine and corticosteroids

***a) Level of CD5 expressed in RA***

Several groups have established that the CD5 B lymphocyte subset may be expanded in patients with RA (Plater-Zyberk et al, 1987, Dauphinee et al, 1988, Youinou et al, 1987, Maini et al, 1987 Taniguchi et al, 1987). Conflicting reports do exist (Sowden et al, 1987) and it has been suggested that RA patients fall into two categories, those with CD5 levels within the normal range and those with elevated levels (Maini et al, 1987).

High levels of this antigen on the surface of B cells is, however, not a prerequisite for the disease since the levels

of CD5 B cells may be independent of disease activity (Brennan et al, 1989, Youinou et al,1990 and Martini et al, 1990). Similarily, it has been shown that from analysis of repeated blood samples, taken from the same donors, the percentage of CD5 B cells were constant over time (Kipps and Vaughan, 1987). However, the number of circulating CD5 B cells appears to correlate with the titre of rheumatoid factor (RF) (Taniguchi et al, 1987). RA patients with RF and anti-nuclear antibody (ANA) have been shown to have associated increased levels of CD5 B cells ( Kazbay and Osterland, 1990 and Becker at al, 1990). A correlation between the titres of IgM RF and the percentages of CD5 was also observed in patients with RA and their family members (Youinou et al, 1990) and in patients with juvenile RA (Jarvis et al, 1992).

***b) CD5 B cell specificity in RA***

Two types of rheumatoid factor (RF), with discrete reactivities, have been described (Burastero et al, 1988).

Polyreactive antibody binding with low affinity to Fc of IgG (similar to antibody produced by CD5 cells obtained from normal controls) and monoreactive RF, binding with high affinity to Fc (expressed by CD5 B cells of RA patients but not normal controls). As, discussed below, a similar situation has been observed in patients with SLE (Casali et al, 1989).

Interesting data, characterising low affinity and high affinity monoreactive RF monoclonal antibodies, generated from circulating CD5 B cells, from a patient with RA, revealed amino acid differences from the germline configuration, of the monoreactive autoantibodies, possibly the result of a process of mutation and clonal selection by antigen (Harindranath et al, 1991).

### 1.4.2.2. Systemic lupus erythematosus (SLE).

SLE is a disease mostly affecting women. Joint pain, skin rashes, fatigue and inflammation are all common but it is the effects on the central nervous system and kidneys which cause the most serious problems. Endocrine, genetic and environmental influences are involved. Antibodies to double-stranded (ds) DNA are regarded as a reflection of the disease and its activity and treatment usually involves combinations of anti-inflammatories, anti-malarial agents, corticosteroids and cytotoxic drugs.

***a) Level of CD5 expressed in SLE***

Some patients with SLE have been shown, not to have elevated levels of circulating CD5 B cells (Plater-Zyberk et al, 1985, Taniguchi et al, 1987 and Dauphinee et al, 1988), while others have reported higher levels than controls (Kazbay and Osterland, 1990, Becker et al, 1990 and Smith and Olsen et al, 1990). In fact, patients had reduced levels of B cells bearing the CD5 molecule after adjustment of steroid doses (Becker et al, 1990 and Taniguchi et al, 1987), therefore, it is likely that the CD5 B cell subset may be sensitive to steroidal drug treatment. Furthermore, two patients have been described (Smith and Olsen, 1990), one with SLE and one with RA, whose percentages of peripheral blood CD5+ B lymphocytes were reduced after therapy, resulting in clinical improvement.

***b) CD5 B cell specificity in SLE***

Clonal analysis of the anti-DNA repertoire of murine B lymphocytes, has shown that antibody forming cell precursors, specific for denatured DNA, are not restricted to the CD5 B cell subset, not even in the case of the autoimmune NZB strain

of mouse (Conger et al, 1987). Furthermore, hybridomas of CD5 positive peritoneal cells, from both Balb/c and NZB mice, could be found to produce Br-MRBC antibodies, but none displayed anti-DNA autoreactivity (Kaushik et al, 1988).

In the human disease, recent data (Suzuki et al, 1990), suggested that in human SLE, there exist two anti-DNA antibody producing B cell subpopulations. One independently secretes anti-DNA (CD5+) and another (CD5-) produces the antibody as a consequence of polyclonal B cell activation.

### 1.4.2.3. Sjogren's Syndrome (SS).

This is a chronic inflammatory autoimmune disorder characterised by a cellular infiltration of exocrine glands, particularly lacrimal and salivary glands. It occurs as a primary ($1^{o}$) event or secondary ($2^{o}$) to other autoimmune diseases like RA, SLE, thyroid disease or myasthenia gravis. There is a marked female to male ratio and an association with HLA-DR1β and HLA-β8. Most patients have ANA with specificity for the nuclear antigens Ro (SS-A) and La (SS-B).

***a) Level of CD5 expressed in SS***

The levels of CD5 in the peripheral blood of patients with $1^{o}$ SS are generally stable over time (Dauphinee et al, 1988). However, once again reports of high numbers of circulating CD5+ B cells in this disease are to be found ( Dauphinee et al, 1988, Youinou et al, 1987, 1988 and Plater-Zyberk et al, 1989).

In SS, autoimmune symptoms frequently precede the appearance of a B cell lymphoma (Kassan et al, 1978). It thus appears that there may be a link between non-organ specific autoimmune diseases and certain lymphoproliferative

disorders, such as CLL. ie, those diseases arising, following a polyclonal B cell activation, such as RA and those following a monoclonal B cell activation such as CLL (Bataille et al, 1988).

Data on the lupus prone NZB mouse supports this view.

### 1.4.2.4. Other incidences of CD5 in autoimmune disease

Elevated levels of CD5 B cells have been observed in children with chronic arthritis (Massa et al, 1988 and Martini et al 1990) and systemic sclerosis (Hardy et al, 1988).

There is also a marked increase in the level found in the blood of patients with Graves' disease (Iwatani et al, 1989). In this disease, the proportion of CD5 B cells was found to be a useful measure of the extent of the hypothyroidism, in that the level of the CD5 B cell subset present in the blood of these patients decreased as the disease responded to therapy.

### 1.4.2.5. Incidences of CD5 B cells in autoimmune strains of mice

i. The main types of lupus mice are, New Zealand black (NZB), New Zealand white (NZW) and their hybrids (NZBxNZW)F1 B/W) and MRL/ lpr/lpr (Theofilopoulos and Dixon, 1985).

A number of the observations made in human RA and SLE parallel the mouse models of autoimmunity, where the depletion of CD5+ B cells is associated with a reduction in disease activity (Smith and Steinberg, 1983).

Although, high numbers of splenic CD5 B cells were reported in NZB and NZW mice (Manohar et al, 1982 and Hayakawa et al, 1983), interestingly, comparable to the human situation, a correlation could be detected between

autoantibody production and levels of CD5 B cells, but not disease activity in these animals (Carmen et al, 1990). In contrast, normal levels of CD5 B cells were detected in MRL/lpr/lpr.

The spleen of old NZB mice have an abnormal population of cells with extra chromosomes, resulting in their having an increased proliferative capacity. These hyperdiploid cells may represent an intermediate stage between autoimmunity and malignancy (Selden et al, 1987).

Other mouse strains genetically programmed to develop autoimmune disease, have been found to have elevated levels of CD5 B cells.

ii. Viable motheaten ($me^v$) or motheaten (me) mice, have severe autoimmune disease and a shortened lifespan (Green and Schultz, 1975 and Rossi et al, 1985). These animals have specifically hypergammaglobulinemia, resulting in high titres of IgM autoantibodies (Sidman et al, 1986). It was demonstrated that hybridomas from splenocytes from $me^v$ mice, yielded higher numbers of monoclonals reactive with autoantigens when compared with other mouse strains (Painter et al, 1988). In fact, flow cytometric analysis of whole lymphocyte populations suggested that the spectrum of autoantibodies expressed by CD5 B cells in normal mice, differs from that in motheaten mice (Mercolino et al, 1988), in that the latter have a low frequency of binding of distearoylphosphatidyl choline (PtC) liposome binding cells than did the normal population.

It is therefore feasable that CD5 B cells from autoimmune mouse strains produce antibodies that differ from those produced by CD5 B cells in normal mouse strains.

iii. Almost all the B cells of the C57BL/6 (me/me) mice are phenotypically CD5 B cells and are found to have high titres of anti-Br-MRBC antibody forming cells (Sidman et al, 1986).

iv A mutant strain, CBA/N, derived from CBA, have an X linked recessive immunological defect (termed xid) at the B cell level (Scher et al, 1982ii). These immunodeficient mice appear to have no detectable CD5 B cells in the spleen (Hardy et al, 1983) or peritoneal cavity (Herzenberg et al, 1986). They do, however, appear to contain CD5 B cell precursors in that they can be reconstituted with autologous bone marrow after treatment with cyclosporin A (De La Hera et al, 1987).

v. RIII/S/J immunodeficient mice are also characterised by having a low anti-Br-MRBC plaque forming cell response and have a reported reduced frequency of peritoneal CD5 B cells (Miernaux et al, 1989) as have the SJL strain (Poncet et al, 1985) which cannot respond to LPS (Hutchings et al, 1986).

vi. Examination of hybrid mice made between me/me and xid suggested that the xid X-linked defect may suppress CD5 B cell development and the associated production of IgM autoantibodies (Scribner et al, 1987).

### 1.4.2.6. Features of CD5 B cells in autoimmunity

Although it has been demonstrated that CD5 B cells may be significantly correlated to the activity of the autoimmune process and further, can be modulated by therapy, in some cases of RA (Becker et al, 1990), whether CD5 B cells are actually responsible for the production of pathogenic autoantibodies in the autoimmune disease, is still a controversial issue.

Whereas, most IgM autoantibodies are produced by CD5 B cells, high affinity pathogenic IgG antibodies are mainly produced by CD5- B cells (Casali et al, 1989). However, CD5 B cells, may be responsible for the pathogenic IgG autoantibody production, by phenotypic switching from CD5+ to CD5- (Shirai et al, 1991).

Very recent reports have revealed, that it would appear, that CD5 (B-1 cells) in the peritoneum, are indeed responsible for autoantibody production and propose the involvment of B-1 cells in autoimmune disease :

These experiments, involved transgenic mice, which were constructed using genes encoding an anti-red blood cell antibody. The B cells created, were almost all specific for an erythrocyte self-antigen (Okamoto et al, 1992). When red blood cells were injected into the peritoneal cavity of these transgenic animals, the result was cell death by apoptosis, of the transgenic B-1 cells. some of the B-1 cells, from the perironeum of the transgenic mice, differentiated into cells secreting antibody at a high rate. This correlated with an observed autoimmune haemolytic anaemia, which rapidly cleared up, as autoantibody secreting cells were removed following injection with red blood cells (Murakami et al, 1992).

It was proposed that a few B-1 cells are spared elimination at the hands of self-antigen and migrate to the peritoneum, where, in the absence of the antigen, can proliferate (De France, 1992).

# Chapter 2

## MATERIALS AND METHODS

## 2.1 CELL SOURCE AND PREPARATION

### 2.1.1 SOURCE OF TISSUE MATERIAL

Peripheral blood was drawn by routine venepuncture into universal containers (Sterilin Ltd, Teddington, UK), containing 200µl of heparin (1000units/ml,CP Pharmaceuticals, UK). This was collected from healthy volunteers, including members of clinical and laboratory staff and healthy medical students, as a source of normal controls.

Patients with rheumatoid arthritis (RA) were defined with classical disease according to The American Rheumatism Association criteria (Ropes et al, 1959). Patient study groups of systemic lupus erythematosus (SLE) and Sjogren's syndrome (SS), were obtained from the Department of Rheumatology, out-patient's, UCMSM, London and diagnosed by classical methods. Chronic lymphocytic leukemia (CLL) patients were selected on the basis of a white blood cell count of at least $20x10^9$/1itre.

Tonsils were obtained from children with chronic inflammation of tonsil tissues. Lymph node and samples of splenic tissue were obtained through intensive care units from accident victims undergoing surgery.

Fetal liver cells were obtained from 21-22 week fetuses and cord blood cells from umbilical cords collected from full-term donors.

## 2.1.2 PREPARATION OF LYMPHOCYTES

### 2.1.2.1 Separation of mononuclear cells

Tissues were gently teased apart in sterile RPMI 1640 (Gibco Ltd, UK) containing 5% heat inactivated (HI) (56°C for 40 mins) new born or fetal calf serum (FCS) (Gibco).

Blood samples were diluted 1:1 in universal containers (Sterilin) with Hank's balanced salt solution (BSS).

The mononuclear cells were in all cases, separated by density gradient centrifugation, according to Boyum et al, 1968. In brief, approximately 12ml of diluted material was layered onto 9ml of Lympho-paque ( density 1.086g/ml; Nyegaard Diagnostica, Oslo, Norway), in universal containers. These were then centrifuged at 400g for 30 mins at room temperature.

The interface cells were removed by aspiration, placed in a universal container containing cold RPMI 1640 (Gibco), supplemented with 5% heat inactivated FCS and washed.

The washing procedure involved a 8-10 mins centrifugation at 400g at 4°C, the cold supernatant was removed, the cell pellet resuspended in cold RPM11640-5% FCS and the washing procedure repeated.

A "pinch" of carbonyl iron (GAS Corporation, New York, USA) was added and the cells rolled at 37°C for 60 mins and the iron containing phagocytic cells were removed by standing the containers on a magnet for a few minutes. Cells in the supernatant were collected.

In some cases the cell preparations were treated by plastic adherence on tissue culture 24 well plates (Costar, USA) for 1 hour at 37°C, to deplete the monocyte population.

The mononuclear cells were then washed three times in BSS containing 5% FCS. The fetal calf serum was inactivated at 56°C for 30 min.

### 2.1.2.2 Counting of cells

Cell viability was always >98%, as determined by staining with acridine orange and ethidium bromide (Sigma, St Louis, Mo), (according to Lee, Singh and Taylor, 1975) and scoring under ultraviolet (UV) microscopy to distinguish viable (green) and non-viable (orange) cells.

Cell counts were performed using an improved Neubauer chamber (Weber, England) and the ethidium bromide/acridine orange stain.

### 2.1.2.3 B cell enrichment :

***a. Preparation of Neuraminidase treated sheep erythrocytes***

Sheep red blood cells (SRBC) (Tissue Culture Services, suspended in Alsever's solution) were washed 4 times with BSS, and resuspended to 5% volume/volume (v/v) in universal containers (Sterilin).

1ml of a filtered, ( 22μm millipore filter), 3mg/ml solution of neuraminidase (Sigma) in BSS was added per 9ml of 5% SRBC and the cells incubated for 1 hr in a 37°C water bath with intermittent inversion of the tubes.

The SRBC were then washed 4 times in BSS and resuspended to 5% v/v in HI-FCS (Gibco) which had previously been absorbed against SRBC. Neuraminidase treated sheep red blood cells (N-SRBC) were stored at 4°C for up to 2 weeks, or until evidence of haemolysis was apparent, whichever period

was the shorter.

*b. Sheep red blood cell rosetting of PBMC*

1 ml of 5% N-SRBC was added per 10ml U-bottomed tube (Sterilin) containing $20x10^6$ adherent cell-depleted PBMC.

The tubes were placed in a 37°C water bath for 10 mins, centrifuged at 400g at room temperature for 7 mins and then placed in an ice bath for 45 mins. The supernatants were then poured off, the cell pellets gently resuspended and 4ml of cold washing medium added.

The cell suspensions were then layered onto 4ml gradients of ice-cold Lymphoprep (density 1.0779/ml, Nyegaard) in U-bottomed tubes (Sterilin) and centrifuged at 400g at 4°C for 15 mins.

0.5 ml N-SRBC were added to each interface and tubes recentrifuged at 400g at 4°C for 10 mins. The interface of B enriched (E-negative) cells was collected, for each tube, washed twice, counted and resuspended at the required concentration in complete culture medium. The B cell fraction was in most cases contaminated by only 1-2% E-rosetting cells.

### 2.1.2.4 Irradiated cells

Adult and cord PBMC preparations were irradiated (40Gy/2500rads) by placing them in a $^{60}$Cobalt source for the necessary time. They were washed once and resuspended at the required concentration in culture medium to be used as feeder cells when required.

### 2.1.2.5 Lymphocyte preservation

Lymphocytes were cryopreserved as follows:

$10^7$ cells were pelleted and resuspended in 0.5ml FCS with 10% DMSO (dimethyl sulphoxide) (BDH Chemicals, UK), in cryogenic vials (Nunc, Roskilde, Denmark). Vials were kept in the vapour phase of liquid nitrogen for 3 hours and then transferred into liquid nitrogen.

### 2.1.3 CELL CULTURE

All cell work was carried out under sterile conditions. Cells were cultured in complete culture medium; RPMI 1640 (Gibco), supplemented with 10% FCS (Gibco), 2mM glutamine (Flow Laboratories, UK), penicillin (100units/ml) (Glaxo Laboratories Ltd, UK) and streptomycin (100units/ml) (Glaxo).

All cell cultures were incubated in a 5% $CO_2$ humidified atmosphere at 37°C.

### 2.1.3.1 Culture with Phorbol myristate acetate (PMA)

Phorbol 12-myristate acetate (PMA) (Sigma) was employed:

i) in the study of the expression and up-regulation of expression of B cell surface CD5 molecules and

ii) to induce immunoglobulin secretion in order to study the specificity of the IgM of individual CLL clones of B cells.

#### *a) Normal control and disease mononuclear cells*

Cells were cultured at $10^6$/ml in 24 well flat-bottom tissue culture plates (Nunc, Gibco, Paisley, UK), in complete culture medium. PMA (Sigma, St Louis, MO) was dissolved in

alcohol at 1mg/ml and added to some cultures to give a final concentration of 10ng/ml.

Cultures were maintained in a humidified 37°C incubator, containing 5% $CO_2$ for 48 hours and harvested for analysis by immunofluorescence. In some experiments, supernatants were collected for analysis of their immunoglobulin content.

Both the concentration of PMA used and the time of the culture were chosen after preliminary experiments on the increase in density of CD5 molecules on T cells with the EPICS C flow cytometer (Coulter Electronics, Luton, UK).

*b) Chronic Lymphocytic leukemic (CLL) B cells*

PBMC were incubated at $2x10^6$/ml in complete culture medium in bulk culture flasks (Nunc, Gibco) at 37°C in 5% C02 for 4-7 days. 10ng/ml of PMA was added to some of the cultures. Supernatants were harvested and their IgM content and light chain restriction, analysed by ELISA.

Some of the supernatants were concentrated 3-5 times by dialysing against Aquacide 111, 20,000 MW (Calbiochem, La Jolla, CA).

### 2.1.3.2 Culture with Epstein Barr virus (EBV)

*a) Preparation of Epstein-Barr virus (EBV)*

Infectious virus was obtained from culture supernatant from the EBV infected marmoset B95-8 cell line (kindly provided by Prof. A. Rickinson, Birmingham).

The B95-8 cells were grown at $10^6$/ml in normal culture medium in a 5% $CO_2$ humidified atmosphere for 1 week and the supernatants collected into 50ml U-bottomed tubes

(Sterilin). Culture supernatant was centrifuged at 300g at 4°C for 10 min and then further clarified by centrifugation for 20 mins. The virus-containing supernatant was then passed through a 0.45mm milipore filter (Acrodisc, Gelman Sciences, UK) to remove any cells from the virus particles. In some cases, to increase the efficiency of the virus, supernatant was concentrated by ultracentrifugation at 30,000g for 3 hours at 4°C. The pellets were then resuspended in cold culture medium and both the concentrate and the untreated supernatants were stored in aliquots at -70°C.

***b) EBV infection and production of cord blood and fetal liver B cell lines/clones***

Cord blood and fetal liver mononuclear cells were cultured in RPMI containing 10% FCS, penicillin and streptomycin (complete medium) at 37°C and 5% $CO_2$. Supernatant from the B95-8 Marmoset line was added at 200μl/$10^6$ cells as the source of Epstein-Barr virus (EBV), (see above). EBV-infected cells (0.2 -100 cells per well) were seeded into 96 well microplates ( Nunc, Roskible, Denmark) in complete medium, together with 2 x $10^4$ irradiated adult PBMC per well.

Often the percentages of B cells were then determined 1-3 days later, using directly conjugated monoclonal antibody Leu 16 (CD20: Becton Dickinson, Mountain View, Calif., USA) and flow cytometry (EPICS C, Coulter Electronics, Luton, UK and a FACSCAN, Becton Dickinson Immunocytometric Systems); method described below.

Plates were generally incubated for 2-7 weeks at 37°C and 5% $CO_2$. Following 10 days incubation, the cells were

fed with 100μl of fresh medium. Supernatants from wells containing growing cells were collected and assayed for IgM using an ELISA.

### 2.1.3.3 Assessment of clonality

Plates containing <33% of the wells positive for growth and IgM secretion, were selected and further assayed for light chain restriction, to determine clonality. Assessment of light chain was performed by indirect immunofluorescence on the B cells and FACScan analysis and by ELISA, both described below.

Those cultures that, satisfied the criteria of the Poisson statistic for limiting dilution assays, for the determination of immunocompetent cell frequencies, and that also secreted IgM, that was found to have only one light chain isotype present, were accepted at this point, to be, in most cases, monoclonal and therefore described as "clones".

This was confirmed by experiments on the differential reactivity of the monoclonal antibody to different imunoglobulin V gene family-associated idiotopes of these "clones". Other growing wells, not consistent with these criteria were called "lines".

Selected B cells in growing wells were passaged into 24 well plates (Costar, Cambridge, Mass., USA) and grown to accumulate cells and supernatants for further analysis.

### 2.1.3.4 Limiting dilution analysis (LDA)

As described by Lefkovits and Waldmann, 1984), LDA was used for the estimation of the precursor frequency of EBV specific CD5+ and CD5- B lymphocytes in cord blood.

Varying numbers of CD5+ and CD5- B cells (0.2, 10, 25, 50 and 100 cell per well) were cultured together with EBV, in the presence of $3x10^4$ irradiated (2500 rads) mononuclear cells in flat-bottomed 96-well microtitre and 60 well Terasaki plates (Nunc), in a final volume of 200 and 50µl, respectively.

Quadruple plates were set up at each cell concentration. The plates were incubated for 6 weeks and an assessment made visually by inverted phase microscopy for the presence of lymphoblasts and areas of active proliferation.

The estimation of the precursor frequency was performed according to Lefkovits and Waldmann,1984. Assuming that antigen responsive cells (ARC) are randomly distributed among the culture wells, the number of ARC per well follows a Poisson distribution expressed in the following function:

$$F(x)=(u^x/x)\ e^{-u}$$

This is the probability of x ARC/well when the mean number of ARC at that cell density is u. The probability of obtaining a culture containing no ARC is given by $Fo = e^{-u}$ and when u=1 Fo is 0.37.

Thus, when N cells per well result in 37% non-responding cultures, the frequency of the ARC is 1/N, which can be calculated by plotting the natural logarithm of the fraction of negative wells/cell concentration, against the number of cells/well plated (Fig.2.1).

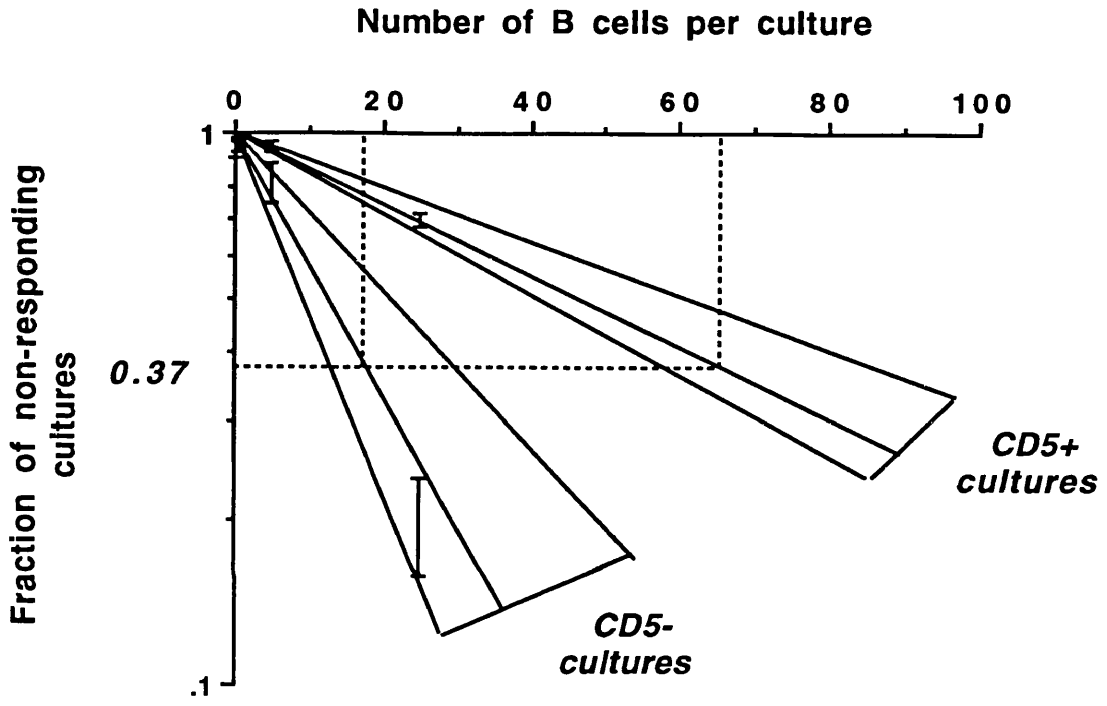


**FIGURE 2.1 : EXAMPLE OF ESTIMATION OF PRECURSOR FREQUENCY OF EBV REACTIVE CD5+ AND CD5- B CELLS BY LIMITING DILUTION ANALYSIS.** The EBV induced proliferation was measured in cultures with 0.2, 1, 5 and 25 responding cells/well in the presence of $3x10^4$ irradiated feeder cells. Quadruple plates were set up for each cell concentration. The frequency of the limiting cell type could be read as the inverse of the number of cells at which 37% of the cultures are non-responding.

## 2.2 PURIFICATION OF MONOCLONAL ANTIBODIES

### 2.2.1 SALT FRACTIONATION AND AFFINITY CHROMATOGRAPHY

In some cases, mouse monoclonal antibodies were salt fractionated using the procedure described in Hudson and Hay, 1980.

Equal volumes of ascitic fluid and saturated ammonium sulphate were mixed, slowly for 30 min. to obtain 50% ammonium sulphate. The preparation was centrifuged at 1500-2000rpm for 15 min. and the supernatant discarded. The precipitate was washed with 40% ammonium sulphate and following centrifugation, redissolved in 0.15M NaCl (saline) and dialysed against the required buffer overnight.

CNBr-activated Sepharose 4B coupled to Protein A (Pharmacia, Uppsala, Sweden) was swollen, washed with PBS and the resulting gel poured into a small column.

Either salt precipitated mouse monoclonal antibody or the ascitic fluid, pre-dialysed against 0.1M Tris/HCl pH8, was applied to the column and equilibrated with the same buffer. Unbound proteins were washed through with the buffer and bound IgG eluted room temperature with 0.1M Tris/HCl pH6.

### 2.2.1 Estimation of protein concentration

The optical density (O.D.) of the collected fractions was read at 280nm and the appropriate fractions were pooled and immediately dialysed against PBS. This method is fully described by Ey et al, 1978. Calculation of the protein concentration was performed assuming an extinction coefficient of 14.3 (1cm cell), for intact IgG (Hudson and Hay,

1980).

In some cases, protein estimation was determined using a Bio-Rad protein assay kit (Bio-Rad, Munich, W. Germany). This is based on the Bradford dye (Coomassie brilliant blue G) binding procedure (Bradford, 1976, Spector, 1978) with bovine serum albumin (BSA) (Sigma) as a standard.

### 2.2.1.2 Separation of $F(ab)_2$ fragments from intact mouse anti- CD5 (IgG) molecules

$F(ab)_2$ fragments of the enzyme digest of mouse IgG were separated from undigested molecules using affinity chromatography on a protein A column, as described above.

Digestion was performed as described by Hudson and Hay, 1980. Purified monoclonal antibodies were dialysed against O.IM sodium acetate pH3.8 and 4.1 for IgG1 and IgG2a subclasses respectively, at 37°C for 3 hours. The protein was then mixed with pepsin (Sigma) at an enzyme: protein ratio of 1: 40.

After 8 hours incubation at 37°C, digestion was terminated by raising the pH to pH7 using 1M NaOH. Finally, the digest was dialysed against PBS and the protein concentration calculated as described above, assuming an extinction coefficient of 14.8 for $F(ab)_2$ of IgG.

The purity and reactivity of the $F(ab)_2$ fragments were assessed by immunofluoresence and flow cytometric analysis.

### 2.2.1.3 Preparation of Fc fragments of human IgG

Fc fragments of Cohn fraction of human IgG were prepared for use in ELISA, as described below. The Fc

fragments were prepared as directed in Hudson and Hay, 1980).

Briefly, from 160mg (20mg/ml) of Cohn fraction 11 (Sigma), digested with the enzyme papain (1.6mg) (Sigma). The mixture was passed over a Sepharose 4B protein A column (Pharmacia, Upsalla, Sweden) and the Fc fragments eluted with glycine HCl pH2.8.

Any undigested material was removed by gel filtration on a Sephacel S300 column (Pharmacia). The Fc fragments were further purified by iso-electric focussing (LKB-8100-1, Bromma, Sweden) using a wide range ampholine carrier (pH3-10) and a sucrose gradient. Fc was isolated at pH7 and dialysed against PBS. The purity of the Fc was established by gel analysis, described below, using antibodies to whole molecule and Fc fragments.

### 2.2.1.4 Determination of purity: *Gel double diffusion (Ouchterlony method)*

The Ouchterlony double diffusion method, (Ouchterlony, 1958) was performed as directed in Hudson and Hay, 1980).

Briefly, a 1.2% agarose gel in barbitonate buffer pH8.6 was melted and poured onto levelled glass slides. The gel was then punched using a template and six small wells (5-10μl capacity) and a large central well (50-100μl capacity) was punched. 60μl of the Fc preparation was added to the central well and 7.5μl of the anti-sera, that is, anti-IgG (whole molecule) and anti-Fc antibodies (Sigma) to the surrounding wells.

The slides were incubated in a humid box at 4°C for 48 hours. A positive control (normal serum) and negative control

(distilled water) were run in parallel.

Formation of precipitation lines was indicative of the purity of the test sample.

### 2.2.1.5 Biotinylation of anti-CD5 antibodies

Antibody was at a concentration of >lmg/ml<20mg/ml and dialysed into 0.25M carbonate buffer at pH9.3. Biotin succinamide ester (Sigma) was made into a stock solution of 1mg/ml in DMSO. 150mg of biotin was mixed per1mg of immunoglobulin for 2-4 hours at room temperature, or overnight at 4°C. The conjugated antibody was then dialysed in multiple changes of PBS overnight and stored at -20°C or at 4°C in 1% bovine serum albumin (BSA) and 0.1% azide.

### 2.2.1.6 Purification of monoclonal IgM

IgM from the B cell clones, in these studies, was purified using ammonium sulphate precipitation followed by affinity chromatography, as described above and in Hudson and Hay, 1989.

The affinity chromatography was performed using a column made with $F(ab)_2$ anti-human IgM (Sigma) coupled to CNBr-activated Sepharose 4B (Pharmacia). This was done, as recommended by the manufacturer, and briefly;

lOmg of protein in coupling buffer (O.IM NaHC03 pH 8.3 and 0.5MNaCl) per ml of gel was mixed continuously for 2 hours at room temperature.

Unbound protein was removed by washing with coupling buffer and any inactive sites blocked by incubation with O.IM Tris/HCl buffer pH 8, for 1 hour.

Finally the mixture was washed with buffers of

alternating pH 4 and pH 8 to remove non-covalently bound protein.

The resulting gel was poured into a small column and equilibrated with PBS.

## 2.3 PHENOTYPIC ANALYSIS OF B CELLS

### 2.3.1 SOURCE OF MONOCLONAL ANTIBODIES

The monoclonal antibodies included in the studies were:

Leu1 and Leul6 (Becton Dickinson, Mountain View, Calif., USA), OKT1 (Ortho Diagnostics, NJ., USA).

UCHTI, UCHT2, 84H10 ($\alpha$-ICAM), TMD3-1($\alpha$-LFA-I) and CD23 (MHM6), were kindly provided by Prof. P.C.L. Beverley, ICRF, London, UK).

RFTI, kindly provided by Prof. G. Janossey, Royal Free Hospital, London, UK).

MID-5, an anti-CDS monoclonal developed in our laboratory (Guarnotta et al, 1984).

Anti-kappa and anti-lambda fluorescein conjugated (FITC) (Coulter, Luton, UK). Affinity purified fluorescein isothiocynate (FITC) conjugated polyclonal $F(ab)_2$ rabbit anti human lambda chains, kindly donated by Prof. Preud'homme, Poitiers University Medical School, Poitiers, France and monoclonal anti-human kappa light chains, kindly donated by Drs M. Fanger and E. Ball, Dartmouth Medical School, NIH, USA).

The majority of the indirect immuno-fluorescence assays were developed using $F(ab)_2$ anti-mouse immunoglobulin (Dako, Glostrup, Denmark).

P3, an irrelevant (IgG1) mouse monoclonal used as a negative control in many of the studies was kindly provided by Dr M Fanger (Dartmouth Medical School, NIH, New Hampshire.

Also used in these studies were the anti-CD5 monoclonal antibodies from the 3rd and 4th International Workshop of "Leukocyte Differentiation Antigens", (#515-532 and 125-145, respectively, as described in the text.

*The monoclonal antibodies used for the studies of immunoglobulin V gene family associated -idiotopes are described in section 2. 7, below.*

## 2.3.2. DETERMINATION OF SURFACE PHENOTYPE BY MICROSCOPY

Enriched B cells ($5x10^5$) were centrifuged and stained in 96 well U bottomed plates (Nunc) for 30min on ice with 50μl of purified kappa or lambda light chain reagents, as described above.

Cells were washed in PBS containing 5% BSA and 0.1% sodium azide and detected by a second layer of FITC conjugated $F(ab)_2$ goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA, USA), absorbed with human Cohn fraction 11 (Sigma, St Louis, MO., USA).

Leul biotin (Becton Dickinson, Calif., USA) binding to B cells was detected using the same method and developed with a second layer of tetramethylrhodamine isothiocyanate (TRITC) conjugated avidin, as described by Guesdon et al, 1979.
Two combinations were used:

(a) FITC-$F(ab)_2$ anti-λ, biotin Leu1, and TRITC-avidin

(b) monoclonal anti-κ, FITC-$F(ab)_2$ anti-mouse IgG.

Normal mouse serum was used to block free binding sites of anti-mouse and prevent binding of second anti-mouse reagent, biotin-Leul and TRITC-avidin.

All reagents were titrated before use and used at plateau concentrations. Control experiments included the use of fluorescent second antibody reagents alone and examination of enriched B cells with UCHTl, a monoclonal recognising CD3

molecules (Beverley et al, 1981).

Fluorescent preparations were viewed with a Zeiss IIIRS microscope with epifluorescence using appropriate filters for FITC and TRITC.

### 2.3.3 FLOW CYTOMETRIC ANALYSIS

Two times $10^5$-$10^6$ cells were placed in U-bottomed microtitre plates (Nunc) and washed three times in washing medium (PBS supplemented with 5%BSA and 0.01% sodium azide).

The cell pellets were resuspended and incubated with 30μl saturating concentrations, (as determined previously by titration experiments), of monoclonal antibodies for 40 min. on ice. Non-specific binding to Fc receptors was blocked with10μl ofl0mg/ml of human immunoglobulins (Cohn Fraction 11) (Sigma, St. Louis, Mo., USA) in PBS containing 1% BSA and 0.01% azide.

After washing twice, the cells were incubated, with 30μl of the fluorescein conjugate of rabbit $F(ab)_2$ antimouse immunoglobulin (Dako, Glostrup, Denmark), diluted 1/50 in washing medium, for a further 40 min on ice.

After washing three times, the cells were fixed in PBS containing 1% formaldehyde and analysed on an EPICS-C cell sorter (Coulter Electronics, Luton, UK) or a FACScan (Becton Dickinson Immunocytometric Systems). At least 25,000 cells were counted for each sample.

### 2.3.3.1 Preparation of CD5+ and CD5- B cell populations

The CD5+ and CD5- cord blood lines/clones in these studies were derived originally from the cell sorting of three, individual cord blood samples.

The enriched B lymphocytes obtained from one of the cord blood samples, was reacted with phycoerythrin labelled Leu 16 monoclonal antibody to CD20 (Becton-Dickinson, Mountain View, Calif., USA) and anti-CD5 antibodies, directed against the proposed Epitopes1 and 2, (Leu1 and UCHT2), visualized with fluorescein conjugate of rabbit $F(ab)_2$ anti-mouse immunoglobulin (Dako), as described in the method above. Also included was a step involving incubation with 3mg/ml of normal mouse serum to block any unoccupied sites and so inhibit non-specific binding of the monoclonals.

The other two cord blood samples used in these studies, were reacted with fluorescein labelled Leu-16 mouse monoclonal antibody to CD20 (Becton-Dickinson) and phycoerythrin labelled Leu-I mouse monoclonal antibody to CD5 (Becton-Dickinson)

After washing, the cells, in each case were sorted on a FACSTAR flow cytometer (Becton-Dickinson Immunocytometric Systems).

Lymphocytes displaying green fluorescence only (CD20+, CD5cells) and those displaying both green and red fluorescence (CD20+, CD5+ cells) were 'gated' and sorted as CD5- and CD5+ B cells respectively.

*This procedure was kindly carried out by Mr Ray Hicks.*

The degree of discrimination achieved in the sorting procedure was then verified by reapplication of the sorted

cells to the FACSCAN (Becton-Dickinson Immunocytometric Systems ) for analysis.

### 2.3.3.2 Blocking experiments

*(used in the epitope mapping studies; see Chapter 3)*

The prototype monoclonal antibodies were Leu1 (CD5; Becton-Dickinson) and UCHT2 (CD5; P.Beverley, ICRF, London, UK). Also used were OKTI (Orthodiagnostics, USA) and RFTI (G. Janossy, London, UK). In the study to map the epitopes of the CD5 molecule the binding of the 3rd and 4th International Workshop CD5 panel were investigated.

Blood mononuclear cells from donors were incubated with pretitrated optimum concentrations of the antibody panels, as described above, washed and biotinylated Leu 1, UCHT2, RFTI or OKTI added together with Avidin-fluorescein.

The cells were read on the FACSCAN flow cytometer to evaluate blocking. The percentage blocking was calculated as a function of the total blocking obtained with unlabelled Leu 1, UCHT2, RFTI or OKTI. That is;

% blocking = (100 - (total %fluorescence with block)/(total % fluorescence with biotin) xlOO)

## 2.4 ANTIBODY ASSAYS

### 2.4.1 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

#### 2.4.1.1 IgM assays

96 well microplates (Nunc, maxisorp, Roskilde Denmark), were coated with 100µl of 1/1000 goat anti human IgM (Tago) in phosphate-buffered saline (PBS) by incubation for 1 hour at 37°C and then 4°C overnight *(stage 1).*

Plates were washed with PBS containing 0.5% Tween (PBS/Tween), blocked with a PBS containing 0.5% bovine serum albumin (PBS/BSA) for 1 hr at room temperature (RT). After washing with PBS/Tween, 100µl of supernatant were added to each well and incubated for 1 hr at 37°C and 1 hr at 4°C *(stage 2).*

Included in the IgM assay was the addition of 100µl per well, of a range of dilutions (0-500ng/ml), of an IgM standard (affinity purified human IgM (Sigma), diluted in the complete culture medium). In order that the final optical densities obtained fell on the linear part of the standard curve, in some cases the test supernatants were diluted.

Plates were washed three times in PBS/Tween, 100µl of $F(ab)_2$ goat anti human IgM alkaline phosphatase conjugate (Sigma) in PBS/BSA Tween containing 0.05% sodium azide were then added and the plates incubated overnight at RT *(stage 3).*

After washing, the plates were developed with 60µl of substrate (p-nitro phenyl phosphate disodium) at 1 mg/ml in ELISA assay mix ($Na_2C0_3$0.05M/$MgCl_2.6H_20$, $10^{-3}$M) for 30 min. at RT. The reaction was stopped by the addition of 60µl1N NaOH. The OD was read on a microELISA reader at 405nm (Dynatech) or on a Titertek multiscan ELISA reader (Flow

Laboratories, Irvine, UK).

Supernatants were tested in duplicate wells, and the mean readings taken, antigen binding activity expressed as a value at 405nm. Supernatants were considered to be positive when the OD values were >mean +2SD above control with culture medium or irrelevant proteins alone and the actual IgM content could be calculated by comparison with the optical densities obtained with the standard preparations of IgM. The threshold for positivity was generally 0.075 (equivalent to the mean value +2SD)

Throughout the assay, each washing step was carried out at room temperature and was repeated three times with 5 min intervals.

All supernatants used for further analysis contained >200ng/ml IgM.

### 2.4.1.2 IgG Assays

96-well microplates were coated with goat antibodies to human IgG (Tago, Burlingame, Calif.,USA) and assays performed as for IgM up to stage 2. The developing reagent was 100µl of 1/1000 dilution of $F(ab)_2$ anti-human IgG alkaline phosphatase conjugate (Sigma, St Louis, USA) and the remainder of the assay was performed as for IgM.

### 2.4.1.3 Kappa and lambda light chain analysis of IgM in supernatants

The assay was the same as for the IgM ELISA up to stage 2. Wells were then washed with PBS/Tween and 100µl added of either 1:1000 goat anti-human kappa or anti-human lambda alkaline phosphatase conjugate (Sigma, St Louis, Mo.,

U.S.A.) in PBS/Tween containing 1%BSA. The plates were then incubated overnight at room temperature. The assay was developed as for IgM.

As an additional control, a selection of positive samples were titrated out, both on plates that were coated with antigen and plates treated with the blocking buffers alone, to account for any non-specific "background" binding.

### 2.4.1.4 Assay for micro-organism specificities

***Binding to K30 polysaccharide of Klebsiella***

*(associated with the 16/6 id, see section 2.7.1.2, below)*

This assay is as described by El Roiey et al, 1987. Briefly, 100μl of K30 antigen in borate buffer was added to microtitre wells (Nunc) and incubated. All incubations were performed for 1 hr at 37°C.

The wells were washed and quenched with 100μl of fetal calf serum per well (Gibco). After washing, 100μl neat supernatants, from the cord blood clones, were added to the wells and incubated.

Following washing, 80μl, 1/1000 dilution rabbit antill antibody, conjugated with alkaline phosphatase (Sigma), was added to each well and incubated. After the final wash, substrate was added and the plates read on an ELISA reader (as previously described above).

The sera from two patients with Klebsiella infection, known to have raised anti-K30 antibodies, diluted 1/100, were used as positive controls.

The supernatants showing an optical density (OD) of >0.15 were considered positive. Negative supernatants ranged from 0.00-0.08 OD.

*These assays were carried out in collaboration with Dr W. Williams (UCMSM).*

## 2.5 DETECTION OF AUTOANTIBODIES

### 2.5.1 AUTOANTIBODY ELISA ASSAYS

#### 2.5.1.1 Rheumatoid factor (RF) assay (anti-Fc of IgG)

This assay was the same as for the IgM ELISA except that stagel involved coating the plates with lOμg/ml of Fc fragments of Cohn fraction of human IgG or with a human IgGl myeloma (a generous gift from Prof. M. W. Fanger and Dr L.Shen).

Supernatants were considered to be positive for rheumatoid factor when the OD values were > mean +2SD above control with culture medium alone. Dose curves were determined for some of the monoclonal antibodies.

#### 2.5.1.2 Collagen type I/II antibody assays

Bovine type I and II collagen (a kind gift from Dr. K. Morgan) were used at 10μg/ml in PBS to coat plates in stage 1 of the assay. The rest of the assay was the same as for the IgM ELISA. Supernatants were considered to be positive when the OD values were > mean +2 SD above controls. Dose curves were made for some of the monoclonal antibodies.

#### 2.5.1.3 Single-stranded DNA (ssDNA) assays

IgM antibodies to ssDNA were detected by ELISA. Assay plates were prepared as follows:

ssDNA was obtained by boiling dsDNA (calf thymus; Sigma) in PBS for 20 min. and then rapidly cooling to 4°C. 96 well microplates were coated with lOOμl of poly-L-lysine (Sigma) at 50μg/ml in distilled water and left at RT for 30 mln. Plates were washed in PBS/Tween and coated with 100μl of calf thymus ssDNA (2.5μg/ml in PBS) and incubated for 3 hr at

RT.

After washing in PBS/Tween,100μl of poly-L-glutamate (50μg/ml in PBS) (Sigma) were then incubated in the wells at RT for 2 hr to block free poly-L-lysine sites. This was equivalent to stage 1 of the IgM ELISA.

After washing the plates 3 times in PBS/Tween, 100μl of supernatants were added and the rest of the assay was identical to the IgM ELISA.

Supernatants were considered to be positive for anti-DNA when the OD values exceeded the mean +2 SD of the negative controls. Dose curves were made for some of the monoclonal antibodies.

### 2.5.1.4 Anti-cardiolipin antibodies

The ELISA used to detect cardiolipin antibodies entailed the coating of 96 well plates with 50μl/well of 50μg/ml of cardiolipin (Sigma) in ethanol and evaporate to dryness under a stream of nitrogen.

Plates were washed with PBS, blocked in PBS containing 10% BSA, 100μl/well for 1 hour at room temperature and washed again in PBS. Supernatants (50μl/well) were incubated for 3 hours at RT.

After washing, 50μl $F(ab)_2$ goat anti-human IgM alkaline phosphatase (Sigma) was added to the plate for 2 hours, washed again and developed with substrate pNitrophenyl phosphate disodium in diethanolamine buffer, pH 9.8, 50μl/well, 1mg/ml, for another 45 min at RT. The reaction was stopped with 50μl /well 3M NaOH and the plates read at 405 nm on a Titertek Multiscanphotometer (Flow).

Samples with OD's> 2SD above the mean of background levels (irrelevant proteins or medium alone) were considered positive. Control sera were kindly provided by Dr.E.N.Harris (RPMS, London).

*These studies were kindly carried out by Prof. P.Y. Youinou, France.*

### 2.5.1.5 Bocking of binding assays

Samples of PBS/Tween containing 1% BSA and 2.5mg of a given monoclonal antibody were mixed with samples of PBS/Tween containing 1% BSA and increasing amounts of soluble IgGl, ssDNA or Collagen types I/II.

After an 18 hour incubation at room temperature, the various mixtures were transferred into ELISA plates precoated with buffer containing lOmg/ml IgGl, ssDNA and Collagen I/II. After a 2hour incubation and subsequent washing with PBS/Tween, the amount of monoclonal antibody bound to the solid phase antigen was measured using an alkaline phosphatase labelled $F(ab)_2$ fragment to human IgM (Sigma), described already.

The binding activity of a given monoclonal antibody observed in the presence of soluble ligand is expressed as a percentage of the binding activity of the antibody measured in the absence of any soluble ligand (100% binding activity).

## 2.5.2 AUTOANTIBODY KITS

### 2.5.2.1 Histone antibody assays

Antibodies to histones 1,2A,2B,3 and 4 were detected using the Nucleoscreen kit (Neosystem).

Briefly, supernatants were incubated on antibody coated plates for 1 hr. at 37°C and washed three times in PBS containing 1% BSA and 0.05% Tween.

Biotin-conjugated $F(ab)_2$ anti-human immunoglobulins were added for another hour at 37°C and the plates washed and incubated with streptavidin-peroxidase for 1 hr. After further washing, ABTS (Boehringer, Mannheim, West Germany) was added as substrate and incubated for 1 hr. at 37°C. ODs were read at 405nm on the Titertek Multiscanphotometer (Flow) and values >2SD above the mean of background levels (irrelevant proteins or medium alone) were considered positive.

*These studies were kindly carried out by Prof. P.Y. Youinou, France.*

#### 2.5.2.2 Thyroglobulin assays

IgM supernatants were tested for antibodies to thyroglobulin by indirect agglutination using an in vitro diagnostic kit, Serodia-ATG (Fujirebio Inc., Tokyo, Japan).

### 2.5.3 AUTOANTIBODY IMMUNOFLUORESCENCE ASSAYS

#### 2.5.3.1 General autoantibody screen

Cryostat sections from rat liver, kidney and human thyroid were cut, dried at room temperature for 2 hours, fixed in acetone for 10 min at room temperature, dried overnight and stored at -20°C until required.

Samples were incubated on such sections overnight at 4°C in a humid chamber. They were then washed twice in PBS for 20 min and incubated with rabbit $F(ab)_2$ anti-human IgM (Dako, Glostrup, Denmark), diluted 1/10 in PBS for 2 hours. After further washing, the sections were mounted in glycerol-based mounting medium containing 25g/l diazabicyclo-octane (DABCO) to prevent fading.

The results were assessed on a Zeiss III RS

microscope using appropriate filters for fluorescein.

*These studies were carried out in The Dept. Immunology, UCMSM*

### 2.5.3.2 Detection of antibodies to tubulin, actin, vimentin, golgi and cytokeratin

Antibodies to vimentin, keratin, actin and tubulin were detected in a cell line IMR-33 derived from a gerbil fibrosarcoma (American Type Culture Collection, Rockville, MD, USA). Cells, cultured in Minimum essential medium 199 with 20% FCS and antibiotics, were seeded onto multispot slides, after brief trypsinization: some cells were pretreated with 20μg/ml colchicine for 18 hrs before use.

After attachment, the cells were washed in PBS, pH 7.4, fixed in methanol at -20°C for 10min, 'rinsed' in acetone for 15 sec at -20°C and thoroughy washed in PBS.

Slides were incubated with test supernatants in a humid atmosphere for 45 min at RT, washed in PBS and incubated for another 45 min in fluoresceinated $F(ab)_2$ goat anti-human IgM. After extensive washing in PBS, slides were mounted in glycerol-based medium containing DABCO and examined on a Leitz Dialux fluorescence microscope using epifluorescence and appropriate filter systems.

Positive controls were commercially available monoclonal antibodies to tubulin and actin (Boehringer).

Positive supernatants stained cytoplasmic arrays of filaments in untreated cells. Antibodies to vimentin were confirmed by the perinuclear pattern of staining following colchicine pretreatment.

*These studies were kindly carried out by Prof. P.Y. Youinou, France.*

### 2.5.3.5 Antinuclear (ANA) antibody and nucleolar

These antibodies were detected by immunofluorescence using Hep-2 cells, as described by Gentric et al, 1990.

*These studies were kindly carried out by Prof. P.Y. Youinou, France.*

### 2.5.4.4 Anti-perinuclear antibodies

These were detected as described by Youinou et al, 1985, using immunofluorescence and human buccal cells.

*These studies were kindly carried out by Prof. P.Y. Youinou, France.*

## 2.6 MONOCLONAL ANTI-IDIOTYPE (ANTI-ID) PRODUCTION

### 2.6.1 PREPARATION OF RF ASSOCIATED MONOCLONAL ANTIBODIES: *C6, C7, 17.109 ($V_\kappa$III associated), G6 and G8 ($V_H$1 associated) B6 and D12 ($V_H$III associated) Lc1, R2.1A2 and 9G4 ($V_H$IV associated).*

Immunisation and fusion protocols, using the murine myeloma line NSO as a fusion partner, have been described in detail ( Lowe et al, 1981 and Mageed et al, 1986). Monoclonal antibodies to idiotypic and light chain subgroup determinants, were produced to two IgM RF paraproteins; Ko ($V_\kappa$IIIb/$V_H$I) from the Wa cross-reactive idiotype family and He ($V_\kappa$III/$V_H$III), as previously described (Crowley et al 1990, Mageed et al 1986 and 1990 and Silverman et al 1988, see Chapter 1).

The immunoglobulin heavy and light chain V gene family and major cross-reactive idiotype (CRI) associations of immunoglobulins recognised by the monoclonal reagents were characterised by ELISA, haemagglutination and Western blot analyses, described in detail by Lowe et al, 1981 and Mageed et al, 1986 and as shown in Table 2.1 and described in Chapter 1.

Monoclonal antibodies C7 and C6 were produced to the IgM RF paraprotein, Ko ($V_H$I), from the Wa cross-reactive idiotype family and monoclonal antibody 17.109 (mouse IgG2b), was produced to IgM RF paraprotein Sie ($V_\kappa$IIIb/VHI), from the Wa CRI family, and kindly provided by Dr. D. Carson (Research Institute of Scripps Clinic, La Jolla, USA), Table 2.1.

Monoclonal antibody Lc1 was prepared by immunization of mice with a mixed cryoglobulinaemia (Ono et

al, 1987) and recognises a CRI associated with the VHIV gene family. R2.1A2 (Evans et al, 1983) and 9G4 (Stevenson et al, 1986), specific for CRI expressed on 90% of IgM paraproteins with cold agglutinin activity. Monoclonal antibody 9G4 was raised in a Lou rat. This is described in Table 2.1 and 2.2.

*All monoclonal antibodies were made available from Dr R.A. Mageed for these studies. In some cases, supernatants from the cord blood clones were tested by him, by ELISA and this is indicated where necessary in the text*

**TABLE 2.1 Characteristics of VHIV associated reagents**

| PROTEIN | ISOTYPE VH-VL subgroup | SPECIFICITY | Reactivity with anti - VH4 CRI | | |
|---|---|---|---|---|---|
| | | | Lc1 | R2.1A2 | 9G4 |
| DIN | IgM - VH4 Vκ3 | Rheumatoid Factor | + | - | - |
| COR | IgM - VH4 Vκ3 | Rheumatoid Factor | + | - | - |
| STR | IgM - VH4 Vκ3 | Unknown | + | - | - |
| CAL | IgM - VH4 Vκ2 | Unknown | + | - | - |
| ODO | IgM - VH4 Vκ3 | Cold Agglutinin | - | + | + |
| HAW | IgM - VH4 Vκ2 | Cold Agglutinin | - | + | + |
| AB26 | IgM - VH4 Vλ | Polyreactive | + | - | - |
| AB17 | IgG3 - VH4 Vκ3 | Polyreactive | - | + | + |
| AB44 | IgA - VH4 Vλ1 | Polyreactive | - | + | + |

Reactivity of monoclonal antibodies, Lc1, R2.1A2 and 9G4, specific for VHIV associated cross reactive idiotopes with IgM paraproteins from the VHIV family of genes.

## TABLE 2.2 Characteristics of idiotope/subgroup reagents

| Mouse McAb. | Association | Paraprotein | CRI family . |
|---|---|---|---|
| G6 | $V_H$I | Ko | Wa |
| G8 | | Ko | Wa |
| D12 | $V_H$III | He | He |
| B6 | | He | He |
| Lc1 | $V_H$IV | --- | --- |
| R2.1A2 | | KAR | --- |
| C7/C6 | $V_\kappa$ III/ IIIb | Ko | — |
| 17.109 | $V_\kappa$ IIIb | Sie | Wa |

Monoclonal antibodies to idiotypic and subgroup determinents were prepared using the IgM RF paraproteins Ko, Sie and He with the cross-reactive idiotype family associations shown.

C7 recognizes a framework group determinent on $V_\kappa$III, whilst C6 reacts with a framework sub-subgroup determinent on $V_\kappa$IIIb.

The other reagents are directed to the idiotopes associated with $V_\kappa$IIIb (17.109), VHI (G6/G8) and VHIII (B6/D12).

VHIV associated reagents, Lc1, R2.1A2 and 9G4 were made using paraproteins isolated from patients with cold agglutinin disease.

## 2.6.2 PREPARATION OF DNA ASSOCIATED MONOCLONAL ANTIBODIES

### 2.6.2.1 Anti-16/6

Antibodies to the CRI 16/6 were obtained by immunizing a rabbit with human monoclonal anti-DNA antibodies (clone 16/6, Shoenfeld et al, 1983). This clone was obtained by fusing blood lymphocytes from an SLE patient with GM4672 parent cells.

### 2.6.2.2 Anti-PR4

Monoclonal antibody to the PR4 id was similarily derived from a human anti-DNA hybridoma, but from a patient with leprosy (Williams et al, 1988).

*Both the monoclonals, 16/6 and PR4, were kindly provided by Prof. D Isenberg, UCMSM for these studies. The assays were carried out in collaboration with Dr W. Williams.*

### 2.6.2.3 Anti-BEG2idβ

Monoclonal anti-BEG-2idβ was obtained by immunizing a rabbit with monoclonal antibody, Beg-2 (Watts et al, 1990). Monoclonal antibody BEG-2 was prepared originally from a 12 week human fetus and binds to dsDNA and ssDNA. The original rabbit polyclonal anti-idiotypic anti-serum was obtained by immunization with monoclonal antibody BEG-2, followed by affinity purification with a pooled human immunoglobulin sepharose 4B column, followed by a BEG-2 sepharose 4B column, as described by Watts et al, 1990. The original BEG-2 id (BEG-2 idα), was shown to be present on the λ light chains of monoclonal antibody BEG-2 by SDS-PAGE and western blotting (described below and by Williams et al, 1988).

In these studies, a second anti-idiotypic antiserum

was used, prepared from a later bleed of the same rabbit and the id identified on the μ heavy chain and designated BEG-2idβ.

*These studies were carried out in collaboration with Dr R. Watts, UCMSM (now Cambridge), who kindly performed a number of the assays, as indicated in the text.*

## 2.7 METHODS OF DETECTION OF IDIOTOPES AND SUBGROUPS

### 2.7.1 FLOW CYTOMETRY

In all experiments, CD5+ and CD5- B cell clones were stained for surface expression of the idiotopes and sub-group determinents (C7, C6, 17.109, G6, G8, B6, D12 and Lc1 ) and evaluated by flow cytometry.

To determine whether or not the density of IgM at the cell surface was sufficient to detect the idiotopes, monoclonal anti-human IgM ( monoclonal antibodies HB-57 and AF6) and anti-human kappa light chains (monoclonal antibodies IgM-67 and 6e1) and anti-human lambda light chains (C4) were used in every sample as a positive control. These were kindly provided bt Dr RA Mageed, Birmingham and are available from UnipathOxoid, Bedford, UK, with the exception of HB-57, which we had available to us in our laboratory.

Idiotope reagents were pre-titrated to determine saturation of binding levels, Fig.2.2.

The method generally, was to incubate $2xlO^5$ cells with 30μl of $F(ab)_2$ antibody, or whole molecule in the case of 17.109 and Lc1 (20μg/ml of purified antibody or ascites1/200, respectively), for 45 min on ice. Cells were washed twice in PBS containing 1% BSA and 0.1% sodium azide. Cells were then incubated for a further 45 min. with 30μl of 1/15 fluoresceinated $F(ab)_2$ rabbit anti-mouse immunoglobulins (Dako). Finally, cells were washed three more times and fixed in PBS containing 1% formaldehyde. Fluorescence was read on a FACScan flow cytometer (Becton Dickinson). Inclusion of irrelevant monoclonal antibody, A7 of the same mouse antibody sub-class as the anti-idiotope and sub-group specific

antibodies (IgGl), excluded binding through Fc receptors.

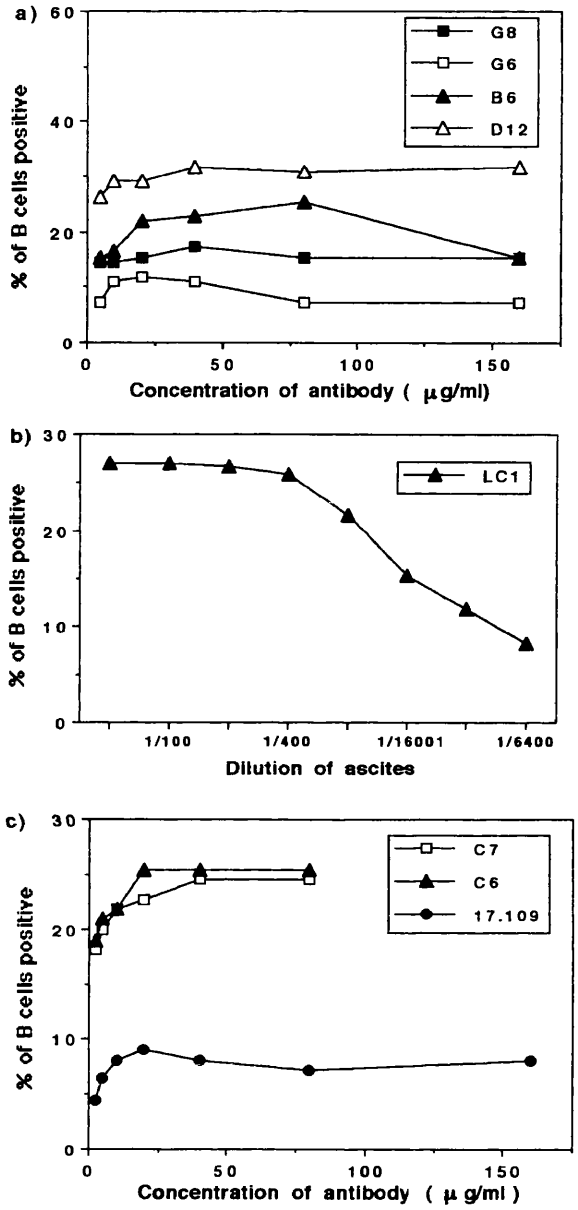


**FIGURE 2.2. DETERMINATION OF OPTIMAL CONCENTRATIONS OF MONOCLONAL ANTIBODIES TO IDIOTYPIC and LIGHT CHAIN SUBGROUP DETERMINENTS**. Dilutions of: $F(ab)_2$ fragments of monoclonal antibodies G6, G8, B6 and Dl 2, (Fig2.2a), ascites fo Lcl (Fig.2.2b) and $F(ab)_2$ fragments of C7, C6 and intact molecule in the case of I17.1O9 (Fig.2.2c).
Results are presented as a percentage of the fluorescent B cells found to be positive determined by surface staining and flow cytometric analysis. In all cases, the MFI was also taken into consideration (not shown).

## 2.7.2 ELISA ASSAYS

### 2.7.2.1 Assay for RF-associated CRI

In order to confirm the findings obtained using flow cytometry (section 2.7.1 above), in some cases, as indicated in the text, ELISA assays were performed with Dr. R. Mageed, using IgM from the culture supernatants collected from the same cells that were stained for surface expression of IgM.

a) Microtitre ELISA plates (Nunc, maxisorp, Roskible, Denmark) were sensitised with purified monoclonal antibodies or their $F(ab)_2$ fragments at 10μg/ml by incubation at 37°C for 2 hr. Since it was not possible to prepare $F(ab)_2$ from mouse IgG2b (Parham et al, 1983), intact molecules of the anti-CRI antibody, 17.109 were used. Monoclonal antibody, Lc1 was an ascites and used at the optimal determined concentration.

Parallel wells were sensitized with negative control monoclonal antibodies OKMI (mouse IgG2b) and G4- $F(ab)_2$ (mouse IgGI) with specificity for CR3 receptor and a private idiotope on Ca ($IgG_2$) paraprotein, respectively. Culture supernatants from CD5+ and CD5- clones were added to the sensitized wells and incubated for a further 2 hr. at 37°C in a humidified incubator with 5% $CO_2$. Bound IgM from culture supernatants was revealed with horseradish peroxidase (HRP) conjugated sheep anti-human μ chain (Binding Site, Birmingham, UK,).

Plates were read as previously described above. Background values of wells containing control monoclonal antibodies and HRP conjugated sheep anti-human μ chain, were automatically subtracted and values significantly higher than the negative controls were considered to be positive.

IgM expressing CRI gave OD values > 0.5 OD compared

with backgrounds of less than 0.05.

**b)** In some of the assays, CRI were detected by sensitizing microtitre ELISA plates (Nunc) with polyclonal sheep anti-human μ chains to bind IgM in the undiluted supernatants from the cultured CD5+ and CD5- clones of B cells.

Purified proteins, positive or negative for the expression of the probed heavy and light chain isotypes and CRI, were included in each assay to confirm specificity. Following three washes with PBS containing 0.05% Tween 20 (PBS/T), murine monoclonal antibodies with specificity for m, k or l (clones AF6, 6el and C4, respectively ; Unipath-Oxoid), or CRI, were added at 1/500 dilutions in PBS/T.

Bound monoclonals were revealed with horseradish peroxidase conjugated sheep anti-mouse or anti-rat immunoglobulins (Binding-Site Ltd). The OD value recorded followed subtraction of background control values.

### 2.7.2.2. Assay for DNA associated idiotypes

**a)** BEG-2idβ

Rabbit anti-id BEG-2β antibodies (0.1mg/ml in 0.1M borate buffer, pH 8.6) were coated onto half the wells (100μl/well) of immunoplates (Nunc), overnight at $4^0$C. The remaining wells were coated with human absorbed rabbit IgG (Sigma) at an equal concentration. After three washings with 0.1M bicarbonate buffer, pH 8.3, non-specific binding was blocked by incubation with 2% BSA in bicarbonate buffer (100μl/well) for 1hr. at 37°C. The plates were then washed three times with PBS containing 0.1% Tween 20.

Culture supernatants from the CD5+ and CD5- clones were applied, undiluted, in duplicate (100μl/well) and incubated for 1hr at RT. Supernatants that gave a positive result on initial screening were titred out. After a further three washes with PBS-T, goat anti-human immunoglobulin (m specific), $F(ab)_2$ fragment, diluted 1/1000 in PBS/T/2%BSA alkaline phosphatase conjugate (Sigma), was applied (100μl/well) and incubated for 1 hour at 37°C.

The plates were then washed four times with PBS/T and once with bicarbonate buffer. The reaction was completed using p-nitrophenyl phoshate substrate (Sigma tablets 104) and the colour read after 1 hour using a Dynatech MR4000 microplate reader at 405nm. The O.D. values on the control rabbit IgG half of the plate were subtracted from those on the anti-idBEG-2, side. The calculated O.D. values were expressed as multiples of the median of the samples tested in each assay. Only those samples >10x median were considered to carry the BEG-2idβ

*These assays were carried out in collaboration with Dr. R. Watts (Cambridge).*

**b) 16/6 idiotype**

Assays of culture supernatants for the presence of the 16/6 id, were performed as described by Isenberg et al, 1984.

Briefly, neat supernatants were used to coat 96-well plates (Nunc) the plates. Plates were incubated for 18 hr at 4°C, then washed three times with PBS-Tween 1%.

The anti-idiotypic serum was diluted 1/12,000 in PBS-Tween (0.1%) and added to the coated wells. After incubation at room temperature for 2 hr, the plates were washed three times and goat anti-rabbit immunoglobulin

serum, conjugated to alkaline phosphatase, was added. The plates were then incubated overnight at room temperature.

The bound alkaline phosphatase was detected by addition of p-nitrophenyl phosphate (1mg/ml in 0.5M NaHCO, 2mM $MgC1_2$, pH 9.5) developing solution. Absorbance at 405nm was read on an ELISA reader, as described above.

Normal rabbit serum (NRS) that had undergone the same procedures, as for the anti-idiotypic antibody was employed as a negative control for the anti-idiotypic serum. On each plate, the sera from 5 healthy individuals served as normal controls. A serum, highly diluted (1/25,000), in borate buffer (0.05M), from a patient with SLE, known to have a high titre of the 16/6 idiotype, was the positive control, run in quadruplicate on each plate. All other determinations were performed in duplicate.

Values for the positive control were generally 0.9 or greater. In contrast, with four exceptions, IgM from the cord blood clones gave values of 0.045 or less.

These studies were kindly performed by Dr. W. Williams, UCMSM.

*c) PR4 idiotype*

Similarily to the 16/6 id assay, culture supernatants were assayed as described by Williams et al, 1988. Briefly, affinity purified rabbit anti-idiotype PR4 (41μg/ml), (prepared as described by Lockniskar et al, 1988), in bicarbonate buffer, pH 9.6, was coated onto 96 well microtitre plates (Nunc) overnight at 4°C. Control wells were coated with normal rabbit serum. After washing three times with bicarbonate buffer and blocking with 2% bovine serum albumin in bicarbonate buffer, for 1 hr at 37°C, 100μl duplicates of supernatants from the cord blood clone cultures, were added.

Supernatants were considered to be positive, if the PR4 idiotype level was above the upper limit of the normal range, which was set at the mean +2 SD of four sera from healthy individuals, included at the time of the assay. This range was virtually identical to the range established on a larger pool of normals (n=30) in a previous study (Williams et al, 1988).

These studies were kindly carried out by Dr W. Williams, UCMSM.

### 2.7.2.3 Inhibition ELISA

BEG-2 idβ positive supernatants from the cord blood clones were further tested for binding site idiotype expression by inhibition of binding to DNA assays.

In these experiments, Immunoplates (Nunc), pre-coated with poly-l-lysine, were coated with ssDNA at 5μg/ml, overnight at 4°C. Reaction mixtures of purified IgM monoclonal antibodies from the cord blood clones and anti-id were pre-incubated for 2 hours at RT, before being added to antigen coated wells for 1 hour at RT. Bound monoclonal antibody was detected by goat anti-human IgM alkaline phosphatase conjugate (Sigma) and the ELISA completed as for the standard binding ELISA, as described above.

*These studies were carried out with the kind help of Dr. R. Watts, Cambridge.*

## 2.7.3 GEL ELECTROPHORESIS

### 2.7.3.1 SDS-PAGE and Western blotting

In some of the studies, the location of a CRI on the IgM molecules obtained from the CD5+ and CDS- clones, recognised by the anti-id monoclonals, was confirmed by sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE) and western blotting.

**a) SDS-PAGE** : The method was originally described by Weber and Osborn, 1969. Proteins were resolved according to the discontinuous buffer system of Laemmli, 1970 on slab gels which consisted of a 10% (w/v) acrylamide in the separating gel and 4% (w/v) acrylamide in the stacking gel.

The final concentrations in the separating gel were as follows: 0.26% (w/v) N,N'-bis-methylene acrilamide, 0.375M Tris(hydroxymethyl)aminomethane-HCL (pH 8.7), 0.06% (w/v) ammonium persulphate, 0.1% (w/v) SDS, 0.06% (v/v) tetramethyl-ethylenediamene (TEMED) in double-distilled water.

The final concentrations in the stacking gel were as follows : 0.13% (w/v) N,N-bis-methylene acrilamide, 0.1 25M Tris(hydroxymethyl)aminomethane-HCL (pH6.8). 0.1% (w/v) ammonium persulphate, 0.1% (w/v) SDS, 0.1% (v/v) TEMED, lOmM EDTA in double distilled water.

The protein samples were dissolved in boiled disruption buffer in a ratio of 1:3, consisting of 0.0625M Tris(hydroxymethyl)aminomethane-HCL (pH6.8), 2% (w/v) SDS, 10% glycerol, 5% (v/v) 2-mercaptoethanol and 0.001% bromophenol blue. They were then boiled for 3 min in a water bath.

10μg of affinity purified IgM was applied and electrophoresis performed, using a multi slab gel apparatus (Bio-Rad) at a constant current of 6mA overnight (approximately 15 hours), as determined by the migration of the bromophenol blue dye marker.

The experimental samples included IgM obtained from the id positive (test sample) and id negative cord blood clone (-ve control) culture supernatants and the id monoclonal antibody (+ve control).

The gel could then be stained with Coomassie blue or blotted onto nitrocellulose membrane.

**b) Western blotting:** Immunoblotting was described by Towbin et al, 1979.

The general method for detection of CRI involves; the proteins from the CD5+ and CD5- clones electrophoresed on SDS-PAGE gels were transferred onto nitrocellulose membrane ( pore size 0.45mm; Schleicher and Schuel, West Germany), using a semi-dry electroblotter (Ancos, Denmark), as described by Kyhse-Andersen, 1984 at a constant current of 0.8mA/$cm^2$ for 3 hours at room temperature.

After electroblotting, the nitrocellulose membranes were cut lengthwise in order to separate the different tracks containing the test samples. At this stage, in order to locate the protein tracks, the nitrocellulose could be stained for protein in Ponceau stain (0.2% (w/v) Ponceau (Sigma) and 3% (v/v) tetra-acetic acid (TCA) in double-distilled water and destained with distilled water.

**c) Immunodetection of proteins** : The nitrocellulose membrane was then cut up and excess protein binding sites are blocked with 3% haemoglobin (BDH chemicals, UK) in PBS and

0.05% Tween 20, overnight at 4°C with constant gentle agitation.

Visualization of the proteins involved incubation with the anti-idiotypic anti-sera, in blocking buffer, followed by incubation on a shaker for 2 hours and washing. The assay was completed using enzyme labelled peroxidase conjugated sheep anti-rabbit immunoglobulin (The Binding Site), and developed in DAB substrate (3,3' diaminobenzidine tetrachloride (Sigma) in Tris/HCl pH7.6, containing $H_2O_2$ (Sigma). The reaction was stopped by washing with tap water. Light chain isotypes of the ids were determined using directly peroxidase conjugated kappa and lambda antibody reagents (Sigma).

## 2.7.4 DETECTION OF EXPRESSION OF VH FAMILY GENES BY BINDING TO STAPHYLOCOCCAL PROTEIN A (SpA).

IgM was tested by ELISA and haemagglutination assay for binding to Staphylococcal protein A (SpA), as described by Sasso et al 1989.

a) **ELISA:** Briefly, 10μg SpA (Sigma) was coated onto microtitre plates (Nunc) at 37°C for 2 hr. Plates were washed and blocked, with 100μl/well PBS/5%BSA. After washing, the test supernatants from the CD5+ and CD5- B cell cultures and the control wells, were added and incubated at 37°C for 2hr, washed and the assay completed as an IgM ELISA, as described above, in section 2.4.1.1.

ELISA measurements from the SpA binding assay are illustrated in Fig.2.3.

b) **Reverse passive haemagglutination assay:** Antibody containing samples of CD5+ and CD5- clone culture supernatants (35μl) was serially diluted in U-bottom microtitre plates (Sterilin, Birmingham, UK) with RPMI containing 2% FCS (Gibco, UK). Antigen sensitised sheep red blood cells (SRBC), as described by Ling et al, 1977, (35μ1 of 0.35% suspension) were added and the pattern of agglutination read after 2 hours at room temperature. Antibody was not applied to the negative control wells.

These assays to investigate the binding of IgM from the CD5+ and CD5- derived B cell clones to SpA, were kindly performed by Dr. R.A. Mageed, Birmingham.

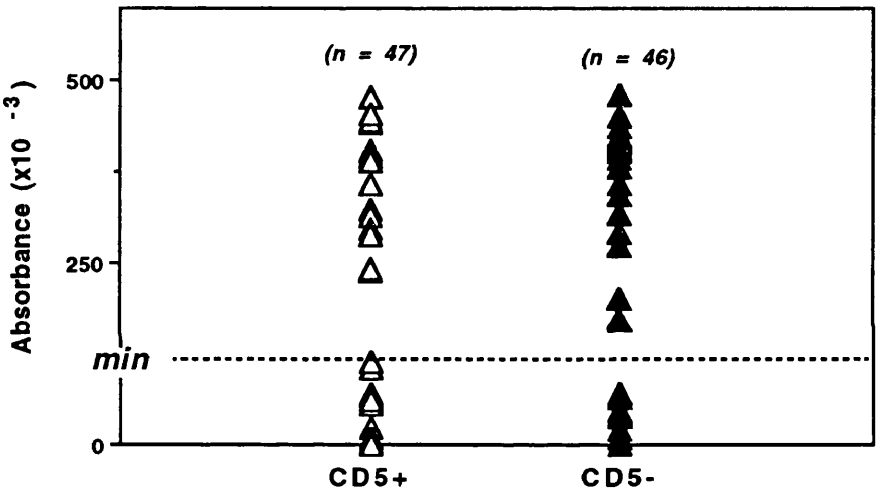


**FIGURE 2.3 IgM FROM CD5+ AND CD5- B CELLS WITH SPECIFICITY TO STAPHYLOCOCCAL PROTEIN A (SpA).** ELISA measurements, expressed as absorbance at 405nm, obtained for the binding of IgM from 47 CD5+ derived clones and 46 CD5+ derived clones, to SpA. Values accepted for positivity are those results of an optical density of ≥0.1. Maximum values obtainable are OD's of 0.5.

## 2.8 STATISTICAL ANALYSIS

Data may be expressed as arithmetic means ±standard deviations (SD).

Comparisons were made by the Mann-Whitney U-rank two tailed test for unpaired data and Wilcoxon's rank test for paired samples.

The chi-square test, with Yates correction was carried out when required.

In some cases, the statistical analysis in these studies, was carried out using the Statview computer software package.

# Chapter 3

## Detection and quantitation of B lymphocytes expressing the CD5 molecule

## 3.1. INTRODUCTION.

One of the main objectives of this study, was to establish the nature of the relationship between CD5 B cell positivity and autoreactivity.

In order to address this issue, initially I had to find an approach that would enable me to successfully detect, isolate and maintain, in culture, the CD5 B lymphocyte subpopulation Iwished to examine.

However, since it had been shown that the level of expression of the CD5 molecule on the surface of B cells was 5-10 times less than the level expressed on T lymphocytes (Hardy and Hayakawa, 1986), it was necessary to develop means of enhancing the detection of this molecule on the surface of human B cells.

**a) Phorbol ester induced enhancement of expression of CD5.**

I had previously shown that the tumour promoter, phorbol myristate acetate (PMA), increased the density of CD5 molecules on the surface of T cells (MacKenzie et al, 1987). I therefore, initially, concentrated on using PMA to attempt to enhance the detection of this molecule on human B lymphocytes.

Since it had been observed that the numbers of CD5 B cells are increased in certain autoimmune strains of mice, (such as New Zealand Black; NZB), (Hayakawa et al, 1983, 1984), the possibility was raised then, that human autoimmune disease, such as Rheumatoid arthritis (RA), might well be characterised by increased numbers of CD5 B cells.

i. As an approach to investigating this, the numbers of CD5 B cells in control tissue and blood, and in blood from patients with RA were determined. Enriched B cell populations were stained with anti-CD5 monoclonal antibodies, Leu1 (Engleman et al, 1981), OKT1 (Reinhertz et al, 1979) and MID-5 (Guarnotta et al, 1984) and assessed for positivity using ultraviolet microscopy. In parallel experiments, PMA was employed to increase the sensitivity of detection of the CD5 molecule.

ii. Furthermore, this study was extended and I similarily analysed the expression of CD5 B cells in the blood of patients with Sjogren's syndrome (SS). These studies were carried out with Prof. PY Youinou, Brest, France.

iii. Finally, I examined the effects of the polyclonal B cell activator, Epstein-Barr virus (EBV), on the expression of the CD5 molecule and tested whether EBV immortalized B lymphocytes carried CD5 molecules before and after PMA treatment.

**b) The use of two anti-CD5 monoclonal antibodies, defining different epitopes on the CD5 molecule.**

Since PMA treatment may modulate the function of CD5 B cells, an attempt was made to increase the sensitivity of detection using a combination of specific monoclonal anti-CD5 antibodies directed to different epitopes on the CD5 molecule.

i. Firstly, I mapped the epitope specificities of a panel of antibodies reacting with the CD5 molecule, using cross

blocking and flow cytometry, using an EPICS V (Coulter Electronics).

ii. I then employed antibodies, to both the two major epitopes we had defined, to increase the fluorescence intensities above those detected using each monoclonal antibody alone.

**c) Use of multiparameter flow cytometry**

During these studies, the laboratory acquired a FacScan (Becton-Dickinson) flow cytometer. This facilitated the analysis of various populations of B lymphocytes using dual immunofluorescence.

i. Enriched B cell populations were stained with monoclonal antibodies, directed against the two CD5 epitopes that had been defined, in combination with a different colour flourescence pan-B cell marker (anti-CD20), (see *Materials and Methods*). The fluorescent cells were then analysed using the FACScan flow cytometer.

ii. Furthermore, I stained B cell populations with red and green fluorescent, directly conjugated monoclonal antibodies, (anti-CD5 and anti-CD20) (see *Materials and Methods*) and also examined these cells using the FACScan flow cytometer.

With these techniques, it was possible to analyse and compare the percentages of CD5 B cells that were to be found in normal peripheral blood samples and those obtained from fresh umbilical cord blood.

## 3.2. RESULTS

### 3.2.1. INCREASED DETECTION OF CD5 POSITIVE B CELLS : phorbol ester mediated enhancement of detection.

A pilot study was carried out, to investigate, the expression of CD5 on B cells derived from normal tissues, followed by an investigation of the expression of this molecule on the surface of B cells derived from patients with autoimmune disease.

In all experiments, the purity of the B enriched cells was assessed by staining with UCHT1, a monoclonal antibody recognising the CD3 molecule (see *Materials and Methods*).

In all cases, except where indicated in the text, the B enriched cell preparations were <5% CD3 positive.

#### 3.2.1.1. Expression of CD5 B cells in normal tissues.

Fig.3.1 shows that few CD5 B cells were detectable in the peripheral blood of normal controls (0.6±0.9%). Higher percentages were found in tonsils (2 ± 0.9%), spleen (2.3±1.5%) and lymph node (2.5±1.3%). Incubation with the tumour promoting agent, phorbol myristate acetate (PMA), (as described in *Materials and Methods*), in the majority of the cases examined, increased, the percentages of CD5 B cells detectable. This was a significant enhancement in the case of peripheral blood ($p < 0.01$).

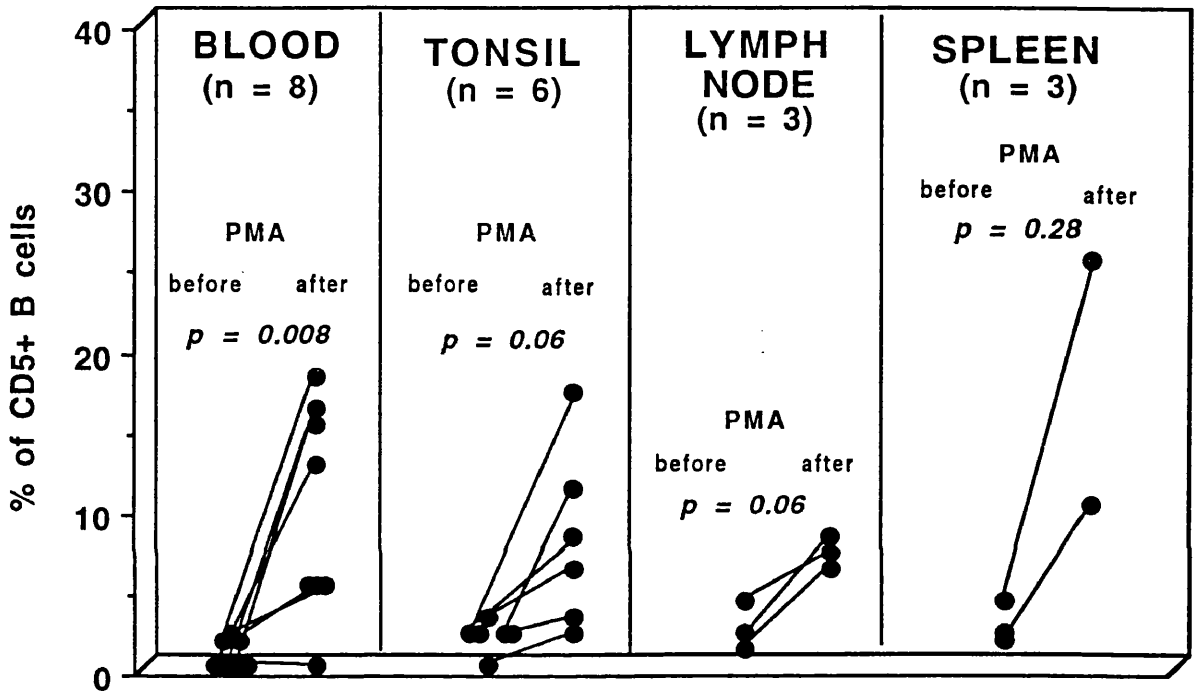


**FIGURE 3.1 : THE EFFECT OF PHORBOL MYRISTATE ACETATE (PMA), ON THE EXPRESSION OF CD5 ON B CELLS FROM PERIPHERAL BLOOD, TONSILS, LYMPH NODES AND SPLEENS.** The percentages of B cells expressing the CD5 molecule in 8 peripheral blood, 6 tonsil, 3 spleen and 3 lymph node samples and the effect of incubation with 10ng/ml Phorbol Myristate Acetate (PMA), (see *Materials and Methods*).

The p value is the result of the comparison of the percentages of B cells expressing CD5 before and after treatment with PMA, for each group.

Enriched B cell preparations, for all groups, were contaminated with <5% T cells, as determined by staining cells with anti-CD3 antibody (see *Materials and Methods*).

### 3.2.1.2. Increased percentages of CD5 B cells in RA blood.

***The clinical characteristics of the patients in this study were as follows :***

The group of patients with RA (one male and 9 female), consisted of 4 with definite RA and 6 with classical disease, according to the American Rheumatism Association criteria (Ropes, M.W. et al 1959). Table 3.1 shows the clinical characteristics of the patients. The mean age was 48 years (range : 26-71) and mean duration of disease 65 months (range : 14-161). 6 patients were sero-positive and 4 sero-negative determined by the latex and modified Rose-Waaler agglutination tests.

Fig.3.2 shows that a higher number of CD5 B cells were detectable in the peripheral blood of patients with RA (1.8±0.8) when compared with those found in the peripheral blood of normal controls (0.5±0.3%), but this was not a significant enhancement. Culture with PMA, however, increased the percentage of CD5 B cells detectable in the blood from both control (13.6±2.4) and patients with RA (28.5±3.8) and the difference between the two groups was statistically significant ($p < 0.01$).

**TABLE 3.1 : Clinical characteristics of Rheumatoid arthritis (RA) patients used in the studies.**

| PATIENT | AGE (years) | SEX | RF * | DISEASE DURATION (months) | TREATMENT † |
|---|---|---|---|---|---|
| 1 | 42 | M | - | 123 | N |
| 2 | 60 | F | + | 49 | N,D |
| 3 | 37 | F | - | 26 | N |
| 4 | 49 | F | + | 67 | N,D |
| 5 | 68 | F | + | 161 | P |
| 6 | 71 | F | + | 123 | P,D |
| 7 | 26 | F | - | 18 | N |
| 8 | 37 | F | - | 30 | N |
| 9 | 30 | F | + | 36 | N |
| 10 | 27 | F | + | 14 | N |

* RF = rheumatoid factor by the latex test
† N = non-steroidal anti-inflammatory drugs ; D = D-penicillamine ; P = prednisone

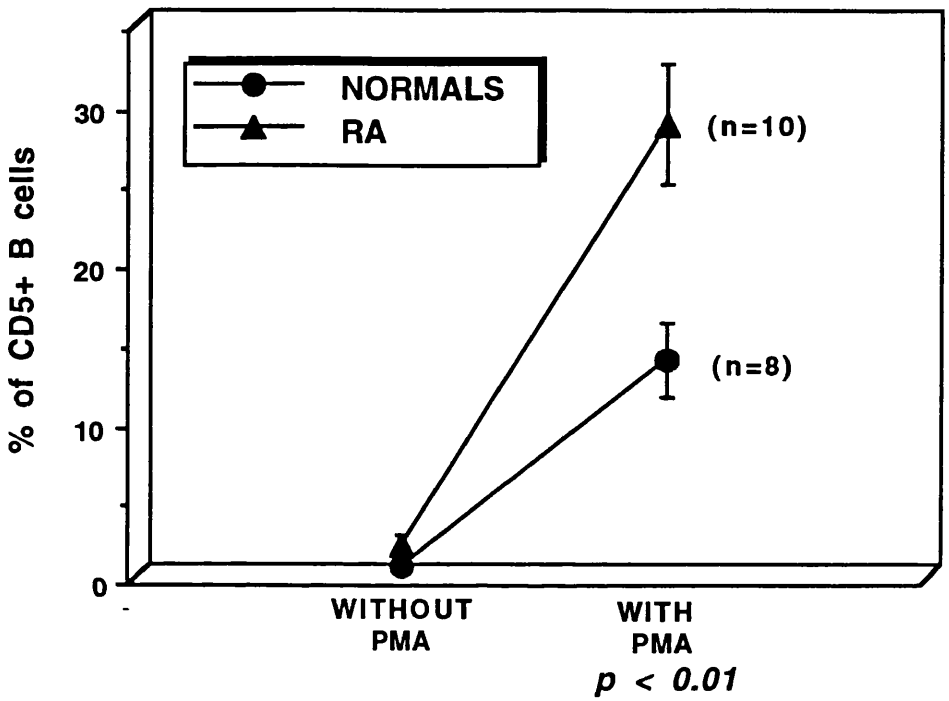


**FIGURE 3.2 : PERCENTAGES OF CD5 B CELLS IN THE PERIPHERAL BLOOD OF CONTROLS AND PATIENTS WITH RHEUMATOID ARTHRITIS (RA), BEFORE AND AFTER INCUBATION WITH PHORBOL MYRISTATE ACETATE (PMA).** The effect of culture with 10ng/ml PMA (see *Materials and Methods*) on the percentage of CD5 positive B cells in the peripheral blood of 8 normal adults and 10 RA patients.

Values are expressed as the mean percentage positive ± the standard deviation (SD). The p value is the result of the comparison of B cells expressing CD5 in the normal and RA groups after enhancement with PMA. Fluorescent preparations were evaluated by fluorescence microscopy (see *Materials and Methods*).

### 3.2.1.3. Increased expression of CD5 on B cells from blood of patients with Sjogren's syndrome (SS).

Table 3.2 shows that very few B cells were detectable in the circulation of normal controls (1.0±0.3%), or in that of patients with SS (1.1±1.6%). Culture with PMA, however, increased significantly ($p < 0.01$) the percentages of CD5 B cells detectable in both groups and furthermore the increase found between the normal controls and the SS patients ($p<0.01$).

**TABLE 3.2 : Percentages of CD5 B cells, in the peripheral blood of normal controls and patients with Sjogren's syndrome, before and after treatment with Phorbol Myristate Acetate (PMA).**

| | Before PMA | | After PMA |
|---|---|---|---|
| **NORMAL CONTROLS** *(n = 11)* | **1.0 ±0.3** | *p < 0.01* | **6.2 ±2.2** *p < 0.01* |
| **SJOGREN'S SYNDROME PATIENTS** *(n = 15)* | **1.1 ±1.6** | *p < 0.01* | **14.5 ±2.8** |

Peripheral blood B cells from normal and patient groups cultured with 10ng/ml PMA (as described in *Materials and Methods*). Values are expressed as mean ± standard deviation (SD). The p value is the comparison of the percentage of CD5 positive B cells before and after treatment with PMA, for each group. Fluorescent preparations were evaluated by fluorescence microscopy (as described in *Materials andMethods*).

### 3.2.1.4 Expression of CD5 on Epstein-Barr virus (EBV) transformed B cells.

Since PMA treatment results in the activation of lymphocytes, it was possible that the small numbers of CD5+ cells observed in controls and especially patients with RA could represent activated cells.

We determined if activation of B cells with EBV, with and without PMA would increase the expression of CD5 on the surface of these cells.

EBV transformed B cell lines, were obtained, by EBV infection of T cell depleted peripheral blood mononuclear cells, from laboratory personnel (as described in *Materials and Methods*). Six such EBV lines, were analysed for surface expression of the CD5 molecule with an EPICS C flow cytometer. Few EBV transformed normal B lymphocytes expressed CD5 molecules either with (1.2±0.5) or without ( 4.1±1.6) culture with PMA (Fig.3.3). The increase in EBV B cells expressing CD5 after PMA treatment was significant ($p < 0.05$).

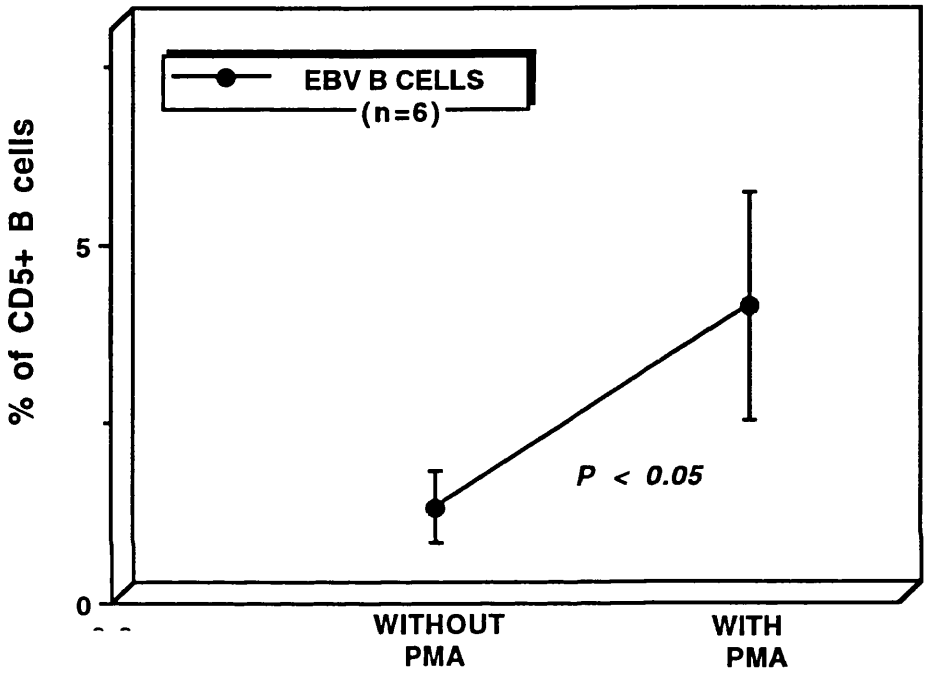


**FIGURE 3.3 : FEW EPSTEIN-BARR VIRUS (EBV) TRANSFORMED B CELLS EXPRESS CD5 MOLECULES, WITHOUT OR WITH TREATMENT WITH PHORBOL MYRISTATE ACETATE (PMA).** Incubation of 6 EBV B cell lines, originating from healthy normal control B cells, with PMA increased the small percentage of B cells that expressed the CD5 molecule on their surface.

Values are expressed as the mean ± standard deviation (SD). The p value is the result of the comparison o the percentage of B cells expressing CD5 before and after treatment with PMA. EBV lines were examined with an EPICS C flow cytometer (see *Materials and Methods*)

### 3.2.2. LIGHT CHAIN ANALYSIS OF CD5 BEARING CELLS.

We examined kappa and lambda bearing human B cells for expression of the CD5 molecule.

B cells carrying either kappa or lambda light chains can express CD5 molecules. No significant differences were seen between the percentages of kappa or lambda B cells expressing CD5, obtained from tonsils, normal peripheral blood, or blood from RA patients (Fig.3.4). No differences were seen following PMA treatment.

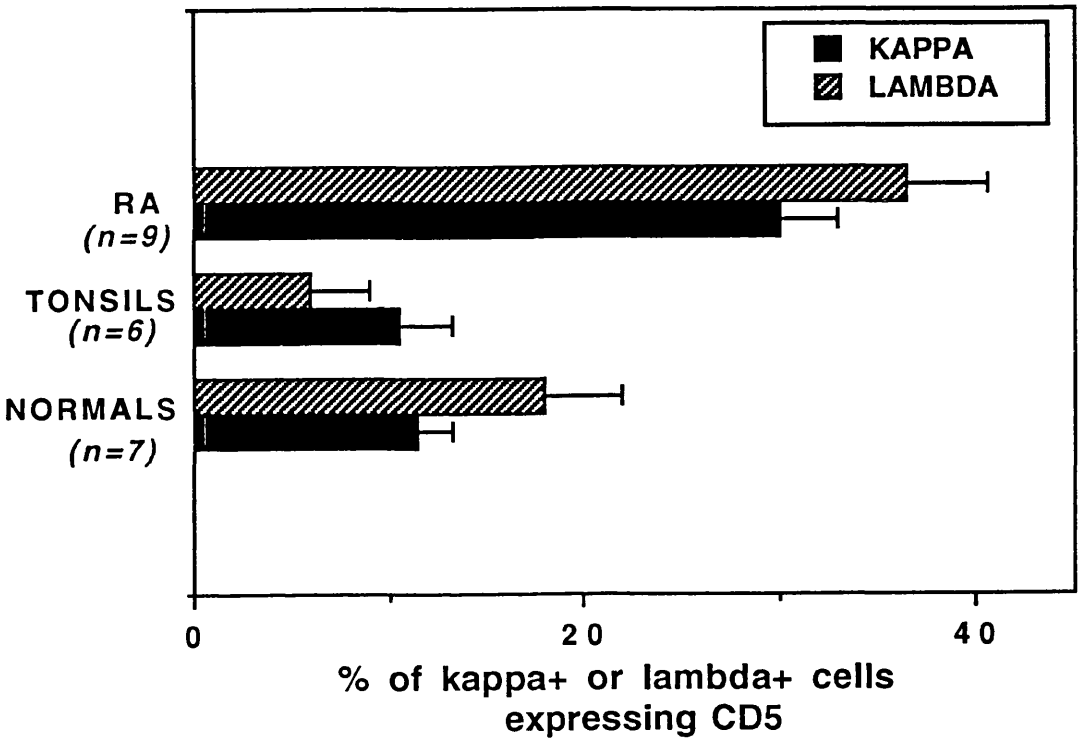


**FIGURE 3.4 : B CELLS BEARING BOTH KAPPA OR LAMBDA LIGHT CHAINS EXPRESS CD5 MOLECULES AFTER PMA TREATMENT.** The percentage of kappa and lambda light chain positive B cells, carrying CD5 molecules in the peripheral blood of 9 patients with Rheumatoid arthritis (RA) and 7 normal controls and from 6 tonsils, after treatment with Phorbol Myristate Acetate (PMA) (see *Materials and Methods*). The percentage of kappa positive or lambda positive cells expressing CD5 are represented as histograms of the mean values for each group. The horizontal lines represent the standard deviation (SD) for each group. Fluorescence was examined using microscopy.

### 3.2.3. EPITOPE MAPPING OF THE CD5 MOLECULE.

Our preliminary studies to enhance the detection of the CD5 molecule on the surface of B lymphocytes, led us next to find a method of identifying these cells, in a way that would not involve the activation of the B cell, as is the result when the cells are treated with phorbol esters.

We attempted to enhance the fluorescence intensity of the CD5 molecule on the surface of human lymphocytes by using a number of different anti-CD5 monoclonal antibodies. To this end, we had available to us, panels of anti-CD5 monoclonal antibodies from the 3rd and 4th Common Leukocyte Antigen International Workshops.

Using an EPICS C flow cytometer to analyse the data, initial studies were performed using the antibodies from the 3rd Workshop to identify epitopes on the CD5 molecule.

#### 3.2.3.1. CD5 monoclonal antibodies used in the study.

Table 3.3 shows the monoclonal antibodies from the 3rd and 4th International Leukocyte Workshops, used in all the epitope mapping experiments described below. Monoclonal antibody numbers (#)515-532 from the 3rd Workshop and #125-145 from the 4th Workshop.

#### 3.2.3.2. Titration of CD5 monoclonal antibodies prior to analysis.

Fig.3.5 a and b shows the percentage fluorescence obtained by flow cytometric analysis, using an EPICS V flow cytometer, of the binding of various dilutions of the CD5 monoclonal antibodies to peripheral blood mononuclear cells from normal adult donors.

Based on these results, all the antibodies were chosen to be used at an optimal dilution of 1/200 except #525 which was used at a dilution of 1/75. Antibodies #130 and 131 proved by poor percentages of binding, not to be anti-CD5 monoclonals and were not included in the study.

**TABLE 3.3 : Monoclonal anti-CD5 antibodies, from the 3rd and 4th International Workshops, used for the epitope mapping studies (section 3.2.3).**

| | MONOCLONAL ANTIBODY NUMBER | ANTIBODY | ISOTYPE |
|---|---|---|---|
| *3rd Workshop antibodies* | 515 | H65 | ? |
| | 517 | 138I | 2a |
| | 518 | UCHT2 | 1 |
| | 521 | BLIb | 1 |
| | 523 | MEM-32 | 1 |
| | 525 | TN71 | M |
| | 526 | 6-2 | ? |
| | 527 | F936D9 | 2a |
| | 528 | OKT1 | 1 |
| | 530 | CRISI | ? |
| | 531 | LEU-1 | 2a |
| | 532 | RFT1 | 1 |
| *4th Workshop antibodies* | 125 | Lo-CD5 | 2a |
| | 126 | T1b | ? |
| | 127 | H154 | ? |
| | 128 | T1c | ? |
| | 129 | A-50 | 2a |
| | 130 | CB01 | 2a |
| | 131 | C1.1E4.7 | 1 |
| | 132 | MF7-14-5 | 2a |
| | 133 | TLP | 2a |
| | 134 | FIII-98 | 1 |
| | 135 | 145-GF3 | G |
| | 136 | F101-15 | 2a |
| | 137 | M-T504 | 2b |
| | 138 | M-T505 | 1 |
| | 139 | M-T503 | 1 |
| | 140 | M-T502 | 1 |
| | 141 | CD5-5179 | 1 |
| | 142 | TM4-1 | 1 |
| | 143 | STBICI | G |
| | 144 | BL6-F4 | 3 |
| | 145 | C156 | 2b |

The number, antibody name and isotype designation of the anti-CD5 monoclonal antibodies (McAb.) from the 3rd (515-532) and 4th (124-145) workshops, used in the studies.

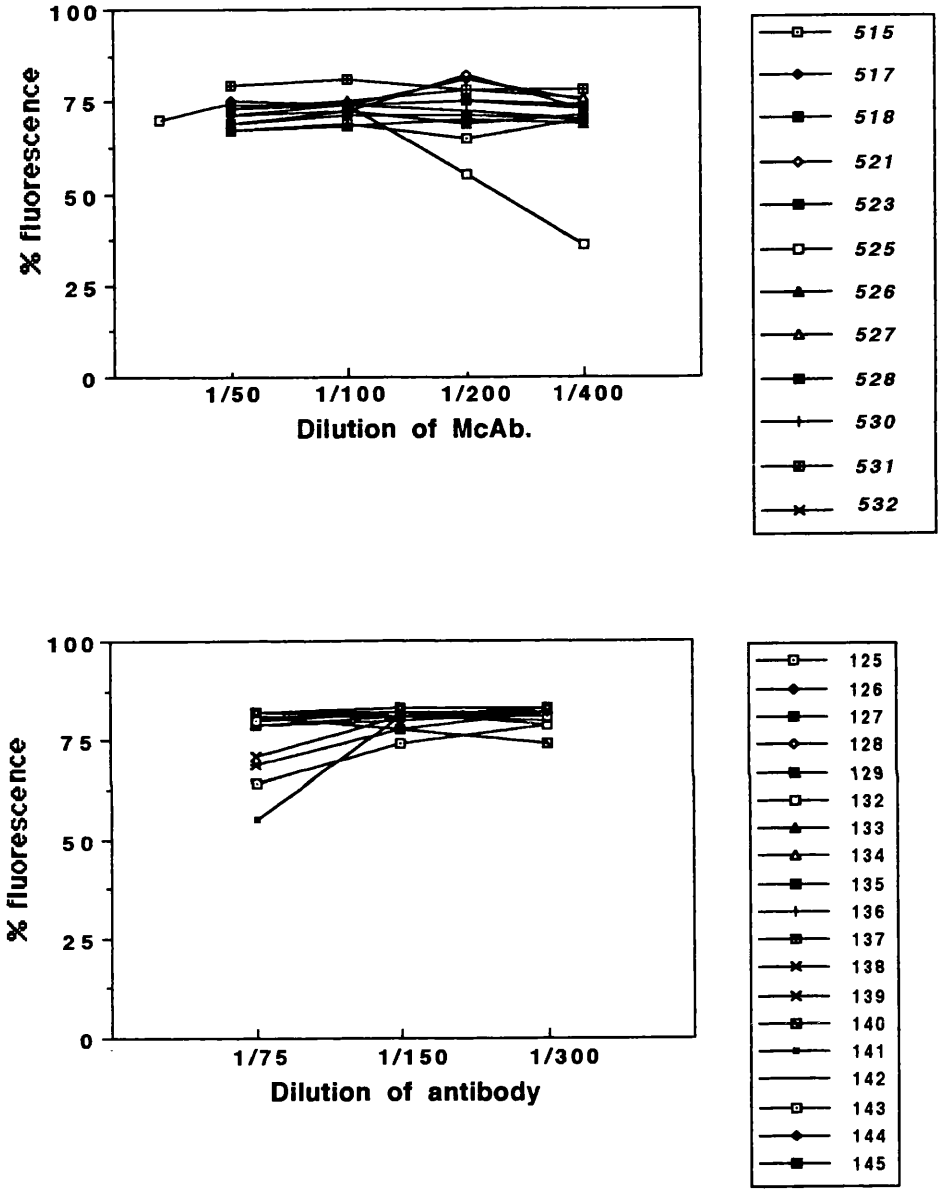


**FIGURE 3.5 : TITRATION OF ANTI-CD5 MONOCLONAL ANTIBODIES (McAb) TO DETERMINE OPTIMUM CONCENTRATIONS.** The percentages of fluorescent cells after staining with different dilutions of the anti-CD5 antibodies from the 3rd (a) (515-532) and 4th (b) (125-145) International Workshops. Fluorescent cells were examined with an EPICS C flow cytometer.

### 3.2.3.3. Cross-blocking of binding of specific anti-CD5 antibodies by the panel of monoclonal anti-CD5 antibodies.

Following biotinylation of purified Leu1, UCHT2, OKT1 and RFT1 antibodies, we determined the mean percentage inhibition of binding to 6-8 normal adult donor peripheral blood lymphocytes, using monoclonal antibodies #515-532 to block the binding. The percentage inhibition was calculated as a function of the total blocking obtained with unlabelled Leu1, UCHT2, OKT1 and RFT1, ( Fig.3.6a, b, c and d respectively) ; see *Materials and Methods.*

We interpreted the high percentages of inhibition of binding obtained by many of the monoclonal antibodies as recognition of the same or, proximal to the same, epitope. Monoclonal antibodies # 515, 521, 523, 527, 526 and 528 bound to similar extents. However, two essentially non-overlapping epitopes were identified. Leu1 (#531), we identified as binding to Epitope 1 and UCHT2 (#518) as binding to Epitope 2. Monoclonal antibody #532 has similar blocking characteristics to LEU1, whereas antibodies #530, 517 and 525 are similar to UCHT2. Fig.3.7 illustrates a schematic epitope map of the CD5 molecule based on these results.

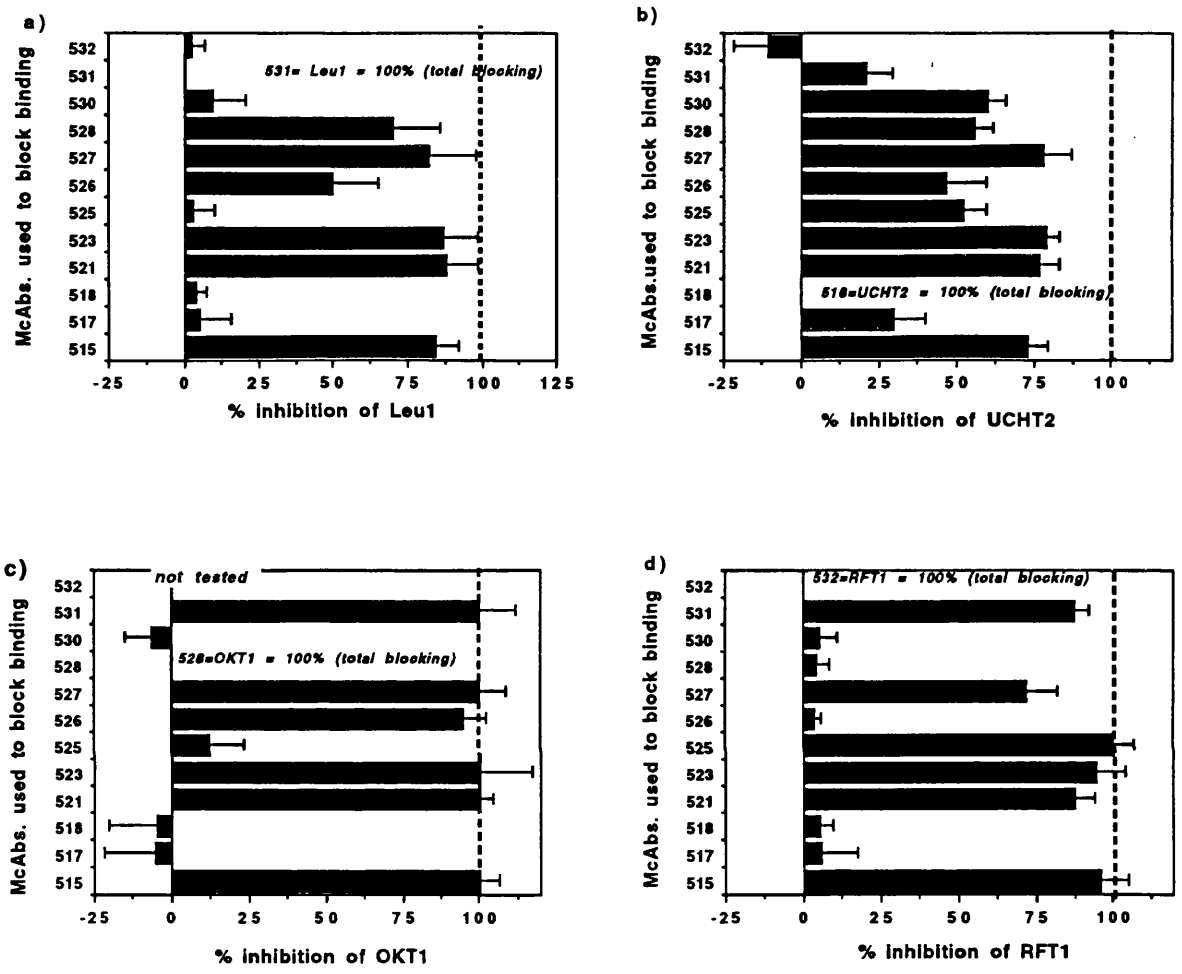


**FIGURE 3.6 : CROSS-BLOCKING OF BINDING OF SPECIFIC ANTI-CD5 ANTIBODIES BY THE 3RD WORKSHOP PANEL OF MONOCLONAL ANTI-CD5 ANTIBODIES (515-532).** The percentage inhibition of binding, to normal peripheral blood mononuclear cells, of Leu1 (a), UCHT2 (b), OKT1 (c) and RFT1 (d), using the panel of anti-CD5 antibodies (515-532) to block the binding, is represented as histograms of the mean percentage of each group. The horizontal lines represent the standard deviation (SD) for each group. Fluorescent cells were examined with an EPICS C flow cytometer. The percentage inhibition was calculated as a function of the total (100%) blocking (see Materials and Methods) (indicated by the vertical dotted lines), for each group.

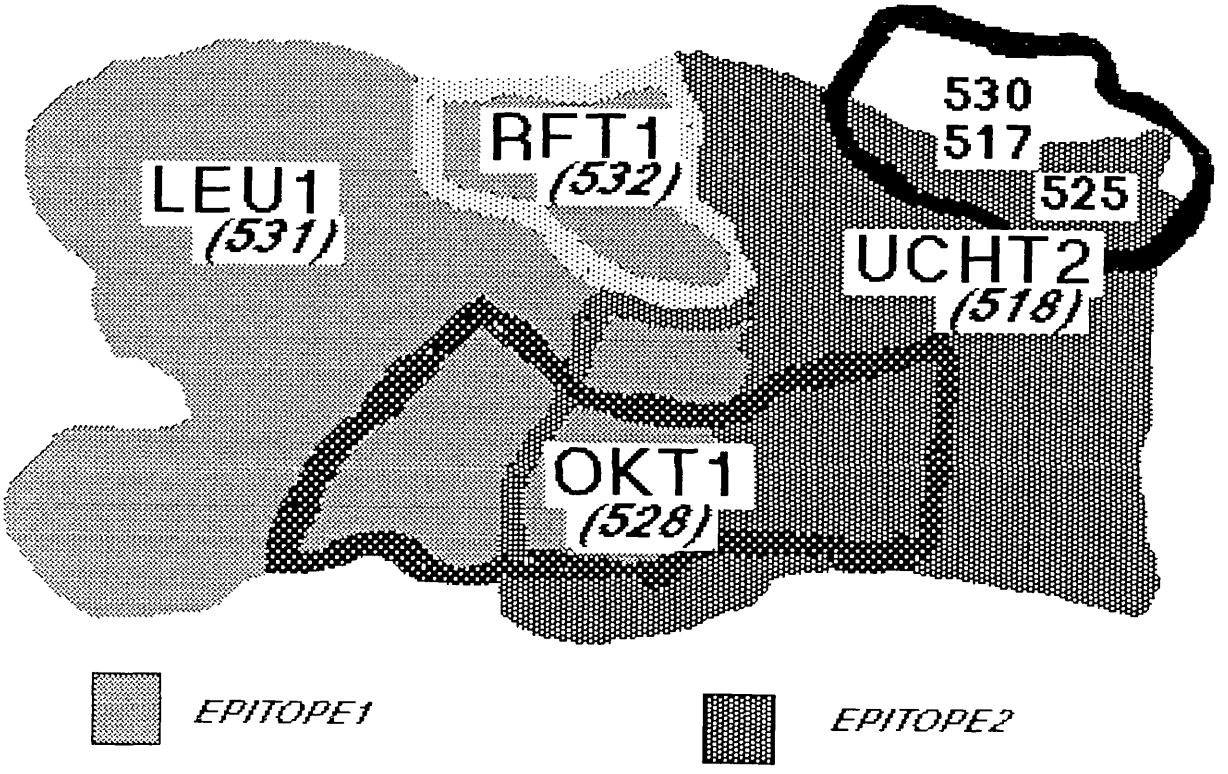


**FIGURE 3.7 : EPITOPE MAP OF THE CD5 MOLECULE.** A proposed epitope map of the CD5 molecule, based on the results of experiments to determine the blocking of binding of Leu1, RFT1, UCHT2 and OKT1 (section 3.2.3.3). Positions of binding of monoclonal antibodies designated 530, 517, 525 are indicated. Two essentially non-overlapping epitopes are indicated ; Leu1 (531) (Epitope 1) and UCHT2 (518) (Epitope 2).

### 3.2.3.4. Enhanced detection of CD5 using combinations of monoclonal antibodies directed against the proposed different epitopes.

Addition of antibodies 531 (Epitope 1) and 518 (Epitope 2), at plateau values, resulted in an increase in the mean fluorescence intensity (MFI) above that detected using each monoclonal antibody alone. This was observed (Fig.3.8) for a) adult T and B cells obtained from 6 donors, b) CLL B cells from 5 donors and c) cord blood non-T, B cells obtained from 7 donors.

Although not quantitative, there also appeared to be an increase in fluorescence intensity, seen using ultraviolet microscopy on fixed cytopreps of the cells.

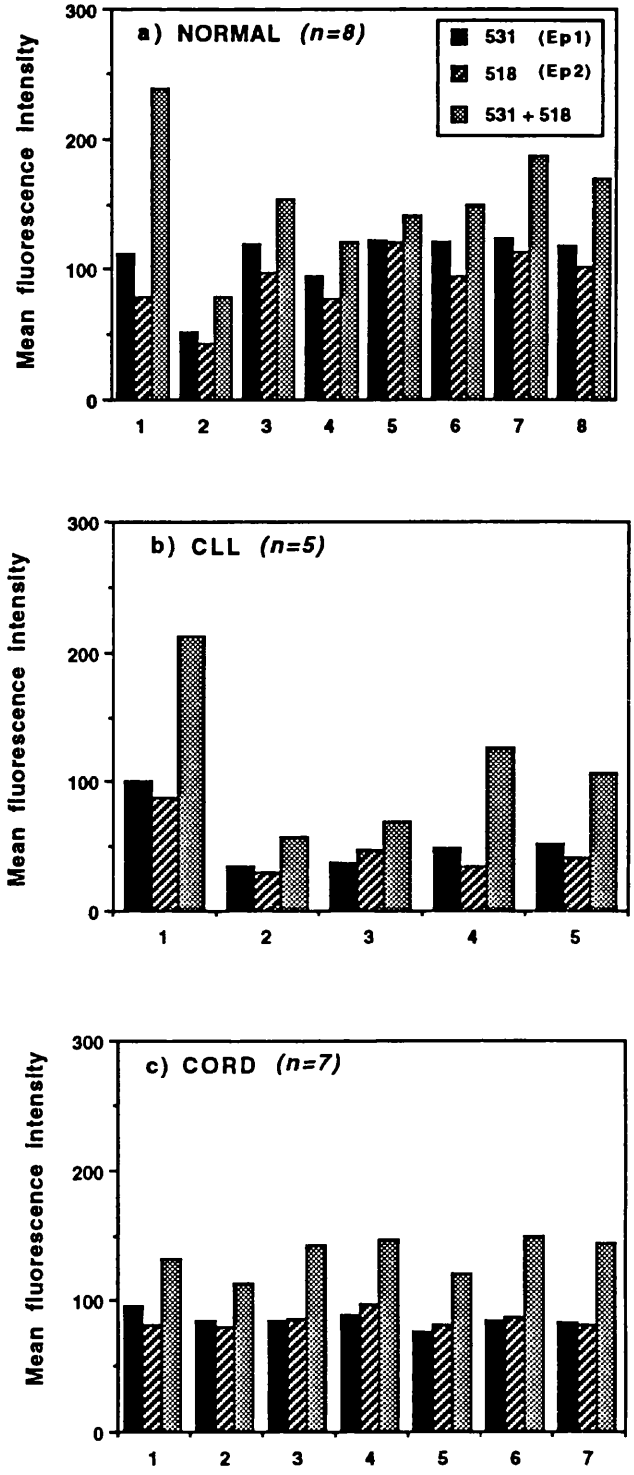


**FIGURE 3.8 : ENHANCED DETECTION OF THE EXPRESSION OF THE CD5 MOLECULE USING A COMBINATION OF ANTIBODIES DIRECTED AGAINST THE PROPOSED DIFFERENT EPITOPES ON THE MOLECULE.** The mean fluorescence intensities of anti- CD5 antibodies, directed against the 2 proposed epitopes (Ep1;531) and (Ep2;518), stained, either alone or together, on adult T and B lymphocytes form 8 donors, (a), Chronic Lymphocytic Leukemic (CLL) B cells form 5 donors, (b) and cord blood B cells from 7 donors, (c), is represented as histograms of the mean fluorescence intensities determined for each group. Fluorescence was detected with an EPICS C flow cytometer.

### 3.2.3.5. Further epitope mapping of panel a of CD5 monoclonal antibodies to the two prototype antibodies, LEU1 and UCHT2.

Having identified two major epitopes, recognised by Leu1 and UCHT2, we then used these antibodies in cross-blocking studies, as described in 3.2.3.3., with a further panel of pre-titrated (Fig3.5b) anti-CD5 antibodies.

Blood mononuclear cells from 5 normal donors were incubated with optimal concentrations of the monoclonal antibodies #125-145, before addition with Leu1 or UCHT2 biotinylated antibodies. The percentage blocking of Leu1 or UCHT2 was calculated as a function of the total blocking obtained with unlabelled Leu1 or UCHT2, as previously determined (3.2.3.3.) Fig.3.9 shows the epitope map with the mean percentage blocking of Leu1 or UCHT2 for each monoclonal antibody. Several of the monoclonal antibodies reacted more with Epitope 1 than 2, #125, 126, 129, 132, 133, 136, 137, 138, 139, 140, 143, 144 and 145. Others, #128, 134, 135 and 142, reacted well with both epitopes. Only one, #141, reacted exclusively with Epitope 2.

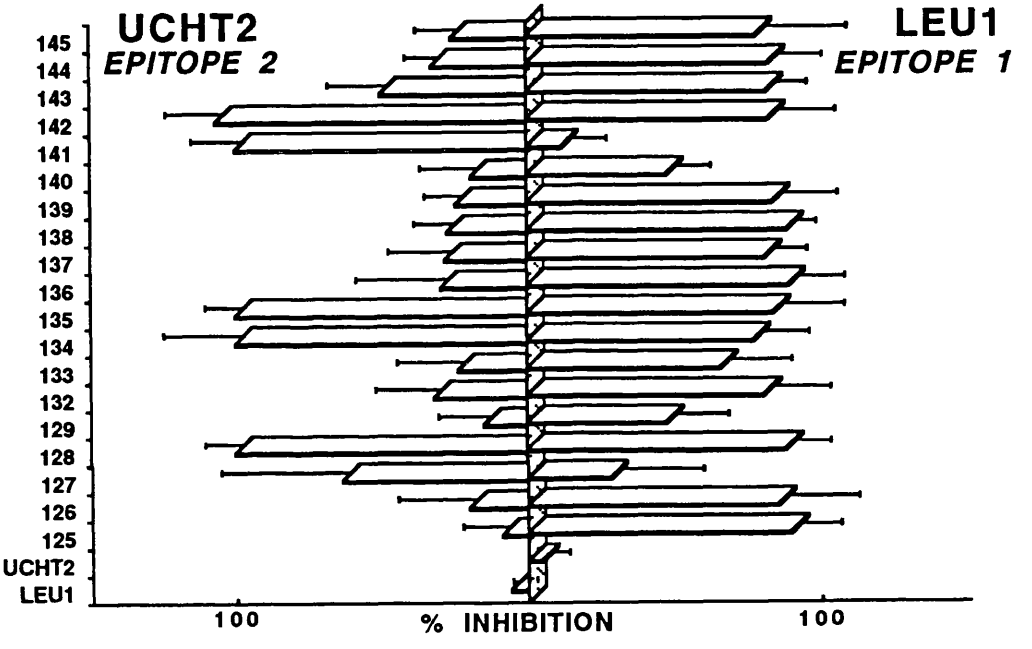


**FIGURE 3.9 : CROSS-BLOCKING OF BINDING OF LEU1 (EPITOPE 1) AND UCHT2 (EPITOPE 2), BY A PANEL OF ANTI-CD5 MONOCLONAL ANTIBODIES (125-145).** The percentage inhibition of binding of Leu1 and UCHT2 to normal adult lymphocytes, by a panel of monoclonal antibodies (125-145) from the 4th International Workshop, is represented as histograms of the mean percentage inhibition for each group. The horizontal bars represent the standard deviation (SD) for each group. The % inhibition was calculated as a function of the total blocking (see Materials and Methods).Fluorescence was determined using an EPICS C flow cytometer.

## 3.2.4. ANALYSIS OF CD5 B CELLS USING TWO COLOUR IMMUNOFLUORESCENCE AND the FACSCAN FLOW CYTOMETER.

At this time, the laboratory obtained a FacScan flow cytometer, with a greater sensitivity than the EPICS C flow cytometer. We then reanalysed the expression of the CD5 molecule on the surface of B cells.

### 3.2.4.1. Expression of CD5 B cells in cord blood and normal adult lymphocytes.

Fig.3.10 shows the quantitation of percentages of CD5+ B cells from 25 cord blood and 20 normal adult donors using FacSCAN analysis.

The peripheral blood T cells from each were depleted using neuraminidase treated sheep erythrocytes (see *Materials & Methods* ) and the enriched B cell populations stained with CD20 fluorescein, (FITC) and CD5 phycoerythrin (PE) labelled markers.

The data shows that a significantly ($p<0.01$) higher number of B cells (60.5%±14.1 ) in cord blood express the CD5 antigen on their surface when compared with normal adults (17%±12.2 ).

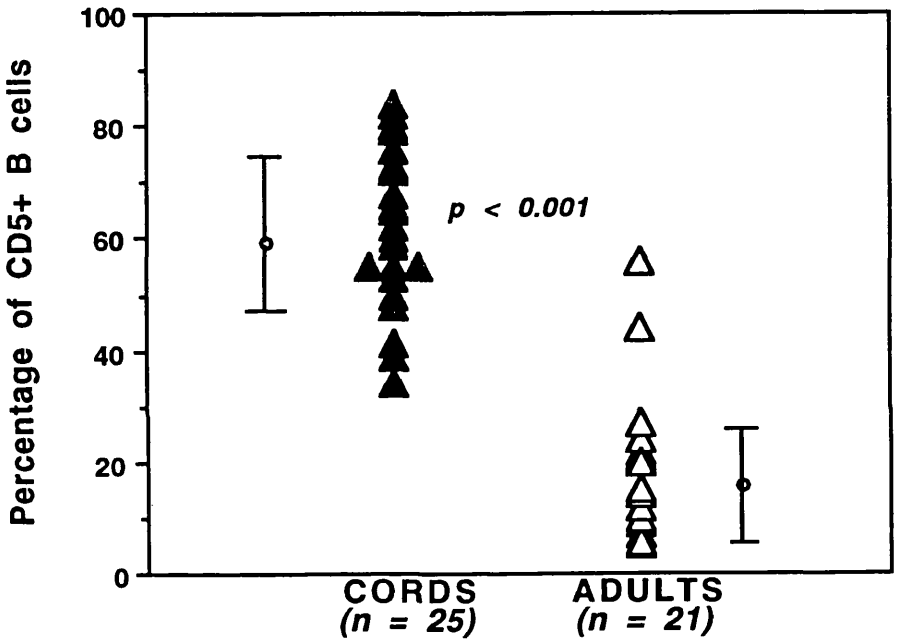


**FIGURE 3.10. EXPRESSION OF THE CD5 MOLECULE ON CORD BLOOD AND NORMAL ADULT B CELLS.** The percentage of CD5 positive B cells from 25 umbilical cord blood and 21 normal adult donors, determined using 2-colour immunofluorescence (as described in *Materials and Methods*). Fluorescence was examined with a FACScan flow cytometer. The dots and vertical bars represent the mean percentage positive ±standard deviation (SD), for each group. The p value is the result of the comparison of the percentage of B cells expressing CD5 in the cord and adult groups.

## 3.3. DISCUSSION

Since the level of expression of the CD5 molecule on the surface of B lymphocytes, was thought to be 5-10 times less than the level expressed on T lymphocytes (Hardy and Hayakawa, 1986), it would require sensitive techniques to identify and characterise this subpopulation of B cells.

In this initial study, we have employed a number of approaches, in an attempt to detect and enhance the detection of the CD5 molecule, on the surface of subpopulations of human B lymphocytes.

**a) CD5 B cells detectable in health and disease.**

The data demonstrated, using ultraviolet microscopy, that CD5 molecules are weakly expressed that CD5 molecules are poorly expressed on tonsil, lymph node, spleen and blood B cells.

Furthermore, a small investigation of the peripheral blood B cells, of a group of patients with the autoimmune diseases, Rheumatoid arthritis (RA) and Sjogren's syndrome (SS), indicated that, they may have increased numbers of CD5 B cells when compared with normal, healthy controls.

These observations extended and confirmed earlier reports of high values for CD5 B cells in RA (Plater-Zyberk et al, 1985). However, some reports, including a further flow cytometric analysis by our laboratory, have found no significant difference between RA and normal, control groups (Sowden et al, 1987, Hannett et al, 1992). This could partly be explained by the criteria with which the diagnosis of RA is based and the exclusion of any allowances for variations in patients' drug treatments and its possible effects on

lymphocyte populations.

It has also been proposed that RA patients fall into two categories, those with CD5 B cell levels within the normal range and those with elevated levels (Maini et al, 1987). Clearly, elevation of this subpopulation alone, is not a prerequisite for this disease.

With regard to SS, other groups have confirmed our findings that CD5 expression is increased on the B cells of these patients (Talal et al, 1987 and Plater-Zyberk et al, 1989).

The elevated level of the CD5 subset in SS is interesting, since this disease, also a chronic inflammatory disorder, may occur as a primary or secondary event to other autoimmune diseases, including RA (Moutsopoulos et al, 1980, Manthorpe et al, 1981, Fox et al, 1984 and Youinou et al, 1987).

**b) Can phorbol ester treatment enhance the detection of specific B cells?**

Previous experiments had shown, that the tumour promoting agent, phorbol myristate acetate (PMA), increased the density of CD5 molecules on the surface of human T cells (MacKenzie et al, 1987).

In this study, we showed that treatment of normal, human blood B cells with PMA, resulted in an improvement in the sensitivity of the detection of the CD5 molecule on the surface of the cell. This confirmed earlier reports of the actions of PMA on B lymphocytes (Miller and Gralow, 1984).

Furthermore, after incubation with PMA, the differences between control and RA blood and control and SS blood were significant.

That PMA treatment results in increased expression of CD5 molecules raises the issue that the CD5 antigen represents an activation marker. We now know that PMA treatment of lymphocytes results in the phosphorylation of CD5 (Lozano et al, 1990) and furthermore, various cytokines have been shown to induce expression of CD5 in CD5- B cells (Freedman et al, 1989, Werner-Favre et al, 1989 and Ying-zi et al, 1991).

However, a recent study in our laboratory, of the regulation of CD5 expression (Paavonen, unpublished), showed that 24 hour stimulation with PMA induced extremely variable changes on the surface, and on the mRNA expression of the CD5 molecule, by chronic lymphocytic leukaemia (CLL) B cells. It is likely however, that CLL cells from individual patients represent different stages in B cell development.

Since all the B cells in the blood, tonsil, lymph node and spleen, examined in this study, do not express CD5 molecules, even after treatment with PMA, the data may be interpretated to mean that, this particular phenotype, if not representing a distinct B subpopulation, may represent a particular stage in B cell differentiation. It is difficult however, to distinguish these two possibilities, although it has been proposed that CD5 B cells could be an immature B cell population (Caligaris-Cappio et al, 1982).

The observation that activation of B lymphocytes by Epstein-Barr virus (EBV), failed to increase the expression of CD5 molecule, is in accordance with other reports, whereby, activation of cells using other common polyclonal activators, pokeweed mitogen (PWM), Staphylococcus aureus (SAC) or anti-IgM, resulted in the down-regulation of CD5 on the cell

surface (Miller and Gralow, 1984), Plater-Zyberk et al, 1988 and Richard et al, 1987).

The results did, however, show that, although few B cells immortalized with EBV, expressed CD5, this could be significantly enhanced, if weakly, by PMA treatment. This would indicate that, the EBV immortalized B cells, included at least some, bearing the CD5 marker. Cloning of these B cell lines might be a way of defining the CD5+ lymphocytes.

Since EBV does not increase the proportion of CD5 B cells, one might conclude, that PMA induces selective proliferation of CD5 B cells or increases the density of CD5 molecules on the membrane of cells already expressing CD5.

In addition, it was interesting that the expression of Ly1, the murine CD5 homologue, might be restricted to lambda bearing B cells (Braun, 1983). We investigated this, in parallel with the main study and described preliminary experiments that would suggest that there is no such bias of light chain expression in the human, even after treatment with PMA.

**c) Is epitope mapping of the CD5 molecule, a better way to identify specific CD5 B cells?.**

Although the CD5 molecule has been gene cloned (Huang et al, 1987) and the structure predicted, little is known about the epitopes recognised by specific monoclonal antibodies.

Initial studies were undertaken to identify more than one epitope on the CD5 molecule, in order to enhance the sensitivity of the detection of this molecule on human B lymphocytes. Using the CD5 monoclonal antibodies from the

3rd International Workshop, two, essentially non-overlapping epitopes, were defined. The prototype monoclonal antibodies were Leu1 (Epitope1) (Becton-Dickinson, described by Engleman et al, 1981) and UCHT2 (Beverley, UCH) (Epitope 2). Further experiments using different CD5 monoclonal antibodies from the 4th International Workshop investigated the binding of these two epitopes.

The data would suggest that there are two major immunogenic epitopes on the extracellular domain of the CD5 molecule, the majority of the antibodies recognising overlapping sequences between these two epitopes.

However, since the identification of these binding sites, was based on the evaluation of blocking assays, clearly the affinity of the individual antibodies under investigation could influence the degree of blocking observed. It is not clear, therefore, if inhibition of binding was a result of steric hinderance, simply because the epitopes were very close together, or because they were truly the same. Although, it *was* possible, to enhance the detection of CD5 on B cell populations of cells using combinations of antibodies directed against the two proposed epitopes. This was observed for normal adult, umbilical cord and CLL blood and was evident when examining the mean fluorescence intensities of the cells by employing flow cytometric analysis. The results were less obvious using microscopy.

**d) Multiparameter flow cytometry to analyse CD5 B cell populations.**

Ultimately, it was ascertained, that analysis of CD5 B cells using two colour immunofluorescence and flow

cytometry, provided clearer quantitation of the percentages of CD5 B cells from various donor blood lymphocytes.

Utilizing these procedures, B cells from a number of normal and disease groups, were examined for the expression CD5 (data not shown).

It was found that umbilical cord blood was highly enriched for the CD5 B cell subset. This was similarily documented by Hardy and Hayakawa, 1986. Based on these observations, I proposed to use cord blood and flow cytometric analysis to further characterise the CD5 B cell.

# Chapter 4

## The Antigen Specificity of CD5+ and CD5- B Cells

## 4.1 INTRODUCTION.

The observations made in Chapter 3, and the results of others, have suggested that, CD5 B cells are increased in patients with Rheumatoid arthritis (RA) and Sjogren's syndrome (SS) (Youinou et al, 1987, Plater-Zyberk et al, 1985 and 1989). Thus, this B cell subset might be of some relevance to autoimmunity.

In an attempt to characterise the CD5 B cell, a study was initiated to determine if there was any difference between CD5+ and CD5- B cell repertoires, with regard to antibody specificity. The two approaches taken were firstly, to use B chronic lymphocytic leukemia (CLL) cells, which express CD5 and secondly, cord blood B cells, which had been shown to be enriched for CD5 B cells (Chapter 3).

**a) Analysis of the antibody repertoire of CD5+ Chronic lymphocytic leukemia (CLL) B cells.**

CD5 was known to be a marker of virtually all malignant clones of B CLL (Wang et al, 1980) and association of this disease with autoimmunity had been reported (Hamblin et al, 1986). These studies, initially explored B CLL, as a model to investigate, the relationship between CD5 positivity and the autoimmune repertoire, at the cellular level.

Using Phorbol myristate acetate (PMA) to stimulate CLL cells, *in vitro*, I determined the specificities of the IgM antibodies secreted, by testing them against a panel of autoantigens.

**b) Analysis of the fine specificities of human neonatal B cells.**

i. In a preliminary study, human fetal liver and umbilical cord blood B cells, known to be enriched for the CD5 B cell subset, were stimulated in bulk cultures or under limiting dilution conditions by Epstein-Barr virus (EBV) transformation. This is an efficient and reliable method, with which to probe B cells for their antibody producing potential.

ii. Since it was important to clarify if, the reactivity of the neonatal B cells, could be soley attributed to their CD5 positivity, cord blood B cells were sorted, prior to cloning with EBV and the resulting clones and lines of CD5+ and CD5- derived B cells, examined for their autoantibody specificity.

iii. To obtain more information on the autoreactivity of the CD5 subset of B cells, the binding patterns of IgM, produced by individual clones, to a number of self-antigens, was examined more extensively. The corresponding avidity, of the IgM from these cells, was investigated using inhibition of binding assays.

## 4.2 RESULTS.

### 4.2.1. THE POTENTIAL ANTIBODY REPERTOIRE OF CHRONIC LYMPHOCYTIC LEUKEMIC (CLL) B CELLS.

Although previous studies by others (Wang et al, 1980), have indicated that B CLL express CD5, a study was undertaken to establish this and show, that there would be little contamination with non-CLL cells in further studies on the IgM secreted by the CLL cells.

#### 4.2.1.1. Phenotypic analysis of the blood lymphocytes of CLL patients.

17 cases of CLL were analysed for surface phenotypes using indirect immunofluorescence and flow cytometry (see *Materials and Methods* ). Blood samples were chosen from patients with white blood cell counts of > $20x10^9$/l, to reduce "contamination" of preparations of malignant monoclonal B cell preparations, with "non-malignant" B cells.

CD3+ cells accounted for no more than 8% and in 82% of the cases, more than 50% of the lymphocytes were CD5+. 10/12 cases expressed kappa light chains and 2/12, lambda light chains, indicating the monoclonality of the cells.

#### 4.2.1.2 Induction of IgM secretion with PMA.

CLL B cells were cultured for 7 days with 10ng/ml PMA and the resulting supernatants collected and analysed for secretion of IgM, by ELISA (see *Materials and Methods*).

28 cases of CLL were initially examined and all but one secreted <100ng/ml IgM spontaneously in the seven day culture system (Table 4.1).

Under the same conditions, the response to PMA resulted in 72% (28/39) of CLL clones examined, secreting a substantial amount (>200ng/ml) of IgM.

Peripheral blood lymphocytes isolated from normal healthy controls could not be induced, under the same conditions, to produce IgM above non-stimulated levels (data not shown).

**TABLE 4.1. Induction of IgM secretion with PMA**

| | CLL * CLONES # 7 day culture | CLL CLONES # 7 day culture with 10ng/ml PMA |
|---|---|---|
| IgM secreted >200ng/ml | 1/28 | 28/39 |

* WBC count > 20 x 1 $0^9$/l

### 4.2.1.3. Light chain restriction analysis

To confirm the clonal origin of the IgM secreted by the CLL B cells, representative samples of the CLL supernatants were examined for their light chain isotypes by ELISA (see *Materials and Methods* ).

The light chain detected in the CLL supernatants corresponded to the cell surface light chain detected by immunofluorescence and flow cytometry in 4.2.1.1, above (data not shown).

#### 4.2.1.4. Reactivity with autoantigens.

Only those CLL B cell cultures that were found to have secreted >200ng/ml of IgM, following treatment with PMA, were examined for autoantigen reactivity.

Supernatants from 26% (8/31) of CLL patients examined, were shown to bind to the Fc of IgG (Fig.4.1). One of these supernatants also bound to ssDNA.

Twelve of these supernatants were further analysed for reactivity with dsDNA, histones 1, 2A, 2B, 3 and 4, cardiolipin, cytokeratin, vimentin, actin, tubulin and binding to tissue sections. (This study was undertaken by Professor P.Y. Youinou, France). Only one supernatant was found with specificity directed towards histone proteins (data not shown). This particular antibody was also polyreactive, in that it also bound to Fc of IgG, ssDNA and dsDNA (Fig.4.1).

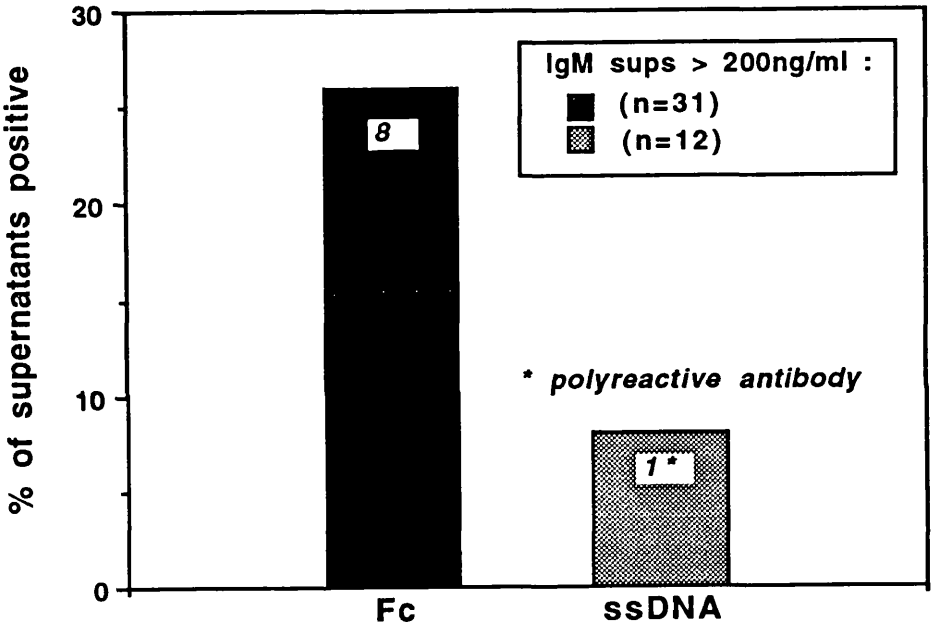


**FIGURE 4.1 THE REACTIVITY OF CHRONIC LYMPHOCYTIC LEUKEMIC (CLL) B LYMPHOCYTES WITH AUTOANTIGENS.** The percentage of 31 supernatants from CLL lymphocytes cultured with PMA for 7 days, that were found to bind to the Fc of IgG and to ssDNA, as detected by ELISA. OD values >2SD above negative controls (medium alone) were considered positive (see Materials and Methods for more details). Each supernatant examined contained at least 200ng/ml of IgM.

* indicates 1 supernatant which was found to be positive for both Fc of IgG and ssDNA, ie, polyreactive.

## 4.2.2. THE ANTIBODY SPECIFICITIES OF FETAL AND CORD BLOOD B CELL LINES/CLONES.

Since our data from Chapter 3 had revealed that neonatal B cells expressed high levels of the CD5 molecule on their surface, (~60% of the B lymphocytes), we felt that such cells would be an appropriate and easily accessible source with which to study further the specificity of CD5 B lymphocytes.

Initial studies were performed on cord blood and fetal liver B cell lines that had been established by immortalization with Epstein-Barr virus (EBV), (see *Materials and Methods* ).

Prior to immortalization, a high frequency of CD5+ B cells was observed in freshly prepared, cord blood (≤60%) and fetal liver (≤80%) B cell samples, used in these particular studies (as detected by immunofluorescence and FACScan analysis, data not shown).

### 4.2.2.1. Cell growth and IgM secretion of EBV transformed cord blood and fetal liver B cell lines.

All microplate wells with growing cells from EBV transformed cord blood and fetal liver, contained detectable amounts of IgM.

In some of the plates, <33% of the wells were positive for growth and IgM secretion. All supernatants that were used for further analysis contained >500ng IgM/ml.

Sixteen of 71 cell lines also had small amounts of IgG detected in the supernatants.

All supernatants were assayed for light chain restriction, by ELISA to determine clonality. By the criteria of the assay (see *Materials and Methods* ), those cultures where

kappa and lambda were both found to be present were described as 'lines'. Supernatants were considered to be monoclonal and are described as 'clones' in cases where only one light chain isotype was detectable and only if the Poisson statistic for limiting dilution also indicated it (see *Materials and Methods*).

### 4.2.2.2 Initial studies on the binding of IgM supernatants from cord blood and fetal liver EBV lines/clones to autoantigens.

IgM antibodies, obtained from EBV immortalized cord blood and fetal liver lines/clones, were examined for their binding to a variety of autoantigens by ELISA. The autoantigens tested included IgG Fc, ssDNA, cardioloipin, Histones 1-4, Collagen types Iand II, thyroglobulin, cytoskeletal components and a tissue section screen (see *Materials and Methods*).

Of 71 IgM (>200ng/ml) containing cord blood EBV line supernatants, 36 bound to the Fc of IgG and 28 to ssDNA (Fig. 4.2a). Similar findings were obtained with 32 fetal liver EBV lines, 16 bound to the Fc of IgG and 27 to ssDNA (Fig. 4.2b).

In this preliminary study, we further examined individual cord blood and fetal liver clones (see *Materials & Methods* ), for their reactivity to autoantigens.

Sixteen supernatants, containing >200ng/ml IgM, from 25 cord blood clones (10$\kappa$ and 6$\lambda$), were positive for autoantibodies, and of these, 7 were polyreactive, (Table 4.2).

IgM from 5/8 fetal liver clones bound to autoantigens and 2 of these was polyreactive. IgM from 40% (10/25) cord blood and 100% (8/8) fetal liver clones bound to tissue sections, (Table 4.2).

Preliminary data showed mainly staining of cytoplasmic components on tissue sections was observed most frequently (data not shown).

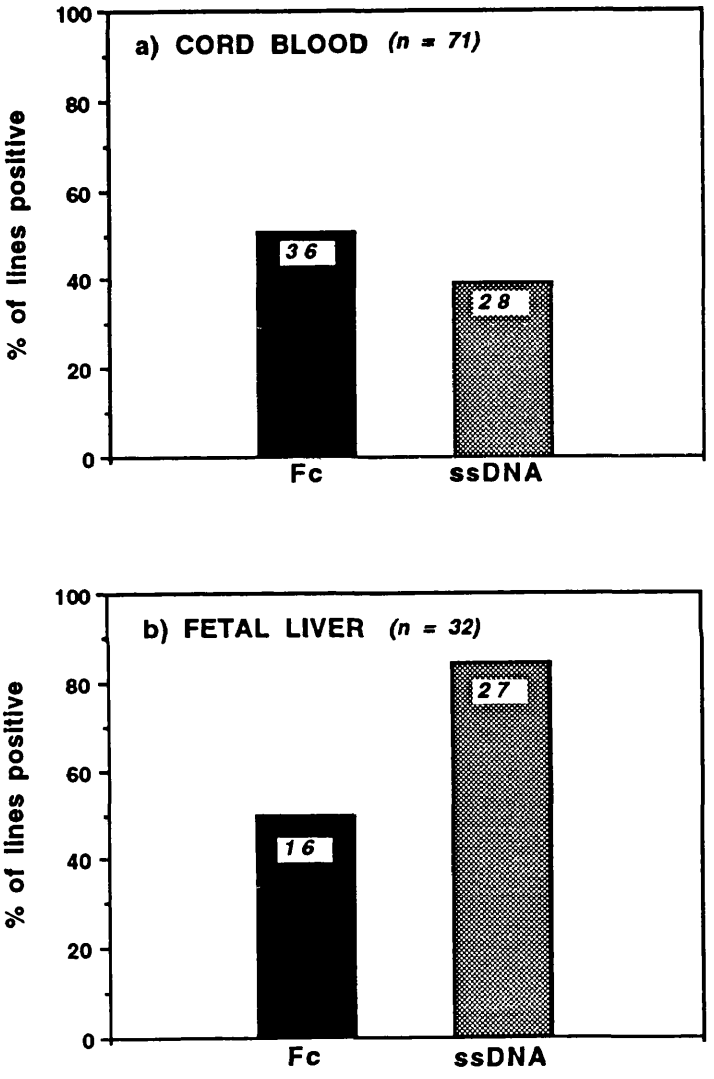


**FIGURE 4.2 ANTIBODY SPECIFICITIES OF CORD BLOOD AND FETAL LIVER EBV B CELL LINES.** The percentage of IgM supernatants collected from 71 cord blood (Fig.4.2a) and 32 fetal liver (Fig.4.2b) EBV B cell lines that bound to Fc of IgG and ssDNA, as detected by ELISA.

**TABLE 4.2 Specificity of cord blood and fetal liver EBV B cell clones.**

| CLONES | | SPECIFICITY | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Fc | DNA | Coll I/II | TG | HIS | VIM | TISS |
| CORD BLOOD | | | | | | | | |
| kappa | 37 | | | | | ■ | | ■ |
| | 46 | | | | | | | ■ |
| | 47 | | | | | ■ | | ■ |
| | 53 | | | ■ | ■ | ■ | | |
| | 55 | ■ | ■ | ■ | ■ | ■ | | ■ |
| | 58 | ■ | ■ | | ■ | ■ | | ■ |
| | 81 | | | | ■ | | | |
| | 86 | ■ | ■ | ■ | ■ | ■ | | ■ |
| | 95 | ■ | | | | | | |
| | 98 | | | | ■ | | | ■ |
| lambda | 38 | ■ | | | | ■ | ■ | |
| | 39 | | | | | | | ■ |
| | 52 | ■ | | | | | | ■ |
| | 94 | | | | | | | ■ |
| | 96 | ■ | ■ | ■ | ■ | | | |
| | 99 | ■ | | | | | | |
| FETAL LIVER | | | | | | | | |
| kappa | 82 | | | | | ■ | ■ | ■ |
| | 83 | | ■ | | | | | ■ |
| | 84 | | | | | ■ | | ■ |
| lambda | 86 | | | | | ■ | ■ | ■ |
| | 89 | ■ | ■ | | | | ■ | ■ |

Binding of cord blood and fetal liver IgM to autoantigens. Individual clones are shown with different patterns of reactivity.

Fc : Fc fragment of human IgG, ssDNA : single-stranded DNA, Col I/II : collagen Type I and II, Tg : thyroglobulin, His : histones 2A, 2B, 3, 4,

Vim : vimentin, Tiss : tissue sections.

## 4.2.3. INITIAL CHARACTERISATION OF CD5+ AND CD5- CORD BLOOD B CELL LINES.

Since preliminary studies had shown that cord blood and fetal liver EBV B cell lines and putative clones can be induced to secrete multispecific or polyreactive antibodies, we wished next to determine whether or not such polyreactivity was restricted to the CD5 B cell subset.

### 4.2.3.1. Positive selection of CD5+ cord blood B cells.

The results described in the following studies, were derived from the cell sorting of 3 individual cord blood B cell samples (as described in *Materials and Methods* ).

Fig. 4.3 shows the gated populations on the cell sorter of one of the three sorts included in this study. In order to get maximum separation between the CD5 positive and negative populations, B cells expressing the highest density of CD5 were sorted from the most negative B cells.

Fig.4.4 shows the analysis of the separation obtained, by reapplying the CD5+ and CD5- sorted populations to the flow cytometer. The purity of the populations was ≥ 98%.

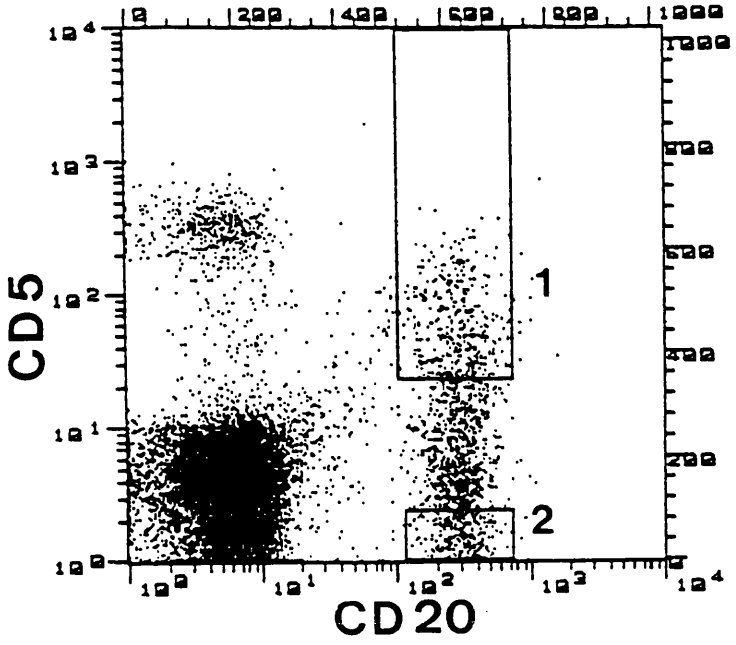


**FIGURE 4.3 PROFILE OF THE GATING CONDITIONS USED TO SORT A REPRESENTATIVE CORD BLOOD B CELL SAMPLE.** Enriched cord B cells stained for CD5 using red fluorescence (vertical axis) and CD20 using green fluorescence (horizontal axis) and applied to a FACStar cell sorter. Lymphocytes displaying green fluorescence only (CD20+, CD5-) and those displaying both green and red fluorescence (CD20+, CD5+), within the rectangles were "gated" and sorted
Gate 1, CD5+ (right sort) ; Gate 2, CD5- B cells (left sort).

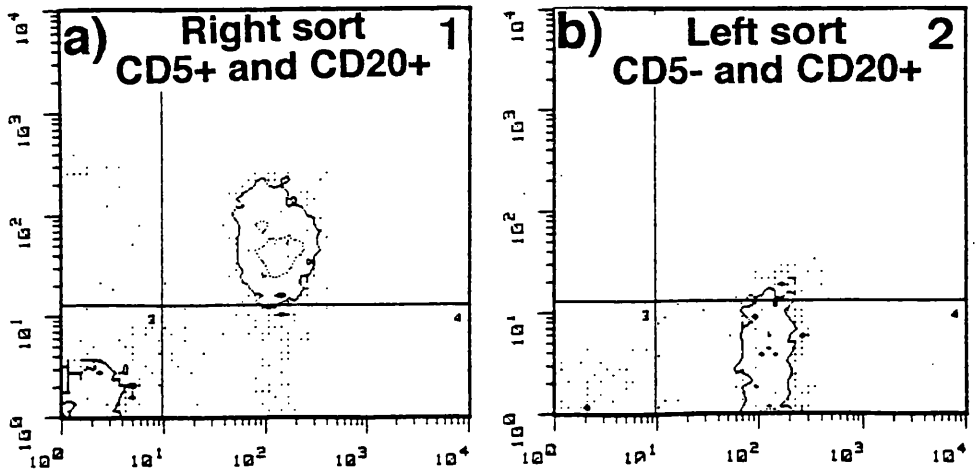


**FIGURE 4.4 THE DEGREE OF SEPARATION OF THE TWO CORD BLOOD POPULATIONS.** The sorting procedure was evaluated by reanalysing the fluorescence labelled cells by a FACScan. CD5+ (a) and CD5- (b) B cells (CD20+), are within the coordinates established for the sorting procedure.

The sorted CD5+ and CD5- derived cord blood B cells were infected with EBV for 24 hours (see *Materials and Methods* ) and cultured as bulk cultures and restricted lines at 100 cells/well in tissue culture plates.

### 4.2.3.2. Immunoglobulin (Ig) secretion by CD5+ and CD5- derived cord blood B cell lines.

A preliminary study was performed to examine and compare the antibody specificities of the two sorted populations of B cells.

Cell lines were established from sorted, EBV transformed cord blood B cells that had been seeded at 100 cells/well. The culture medium was removed and replaced with fresh medium at the end of the first and second weeks of culture, to eliminate IgM secretion from the feeder cells. Supernatants for examination, were collected after 6 weeks of culture, post EBV infection were assayed initially for IgM, IgG and light chain isotype content, by ELISA (see *Materials and Methods* ).

i Virtually all the cell lines generated from both CD5+ and CD5- cord blood B cells secreted both kappa and lambda light chains and detectable (in fact, >500ng/ml) IgM into the supernatants. No detectable (<50ng/ml, the minimum detectable within the limitations of the ELISA) IgG was observed.

i i A higher mean secretion of IgM (23$\mu$g/ml±6.17) was observed for the CD5- derived EBV than was observed for the CD5+ (10$\mu$g/ml±7.56) lines, after 6 weeks of culture of 100cells/well, as shown by Fig.4.5.

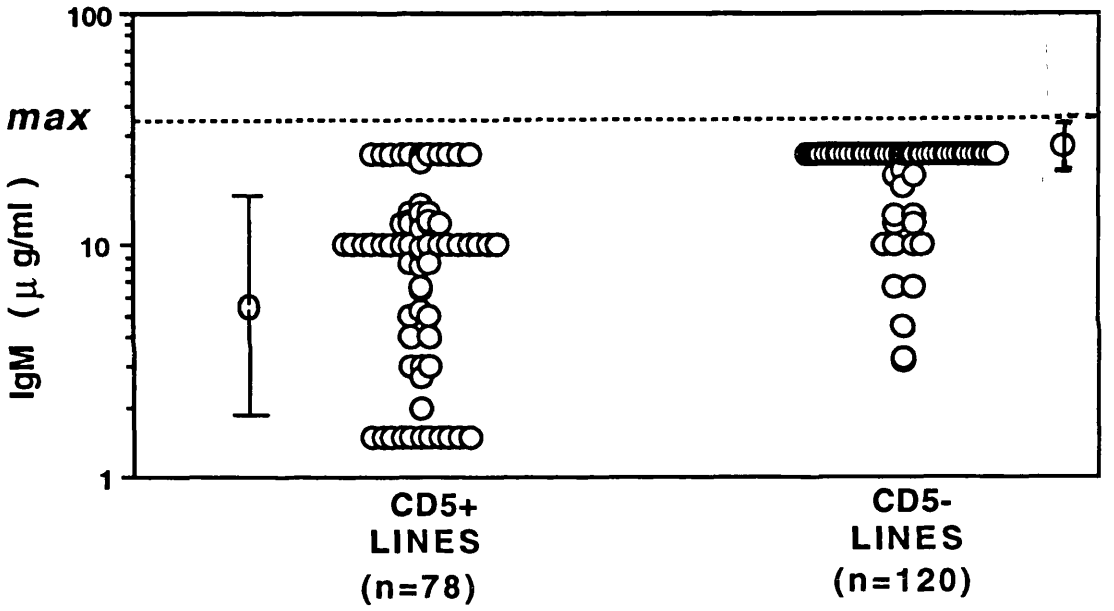


**FIGURE 4.5. IgM SECRETED BY CD5+ AND CD5- CORD BLOOD EBV LINES.** The concentration of IgM (μg/ml) obtained by examining supernatants, collected from 78 CD5+ and 120 CD5- fractionated, EBV infected, lymphocytes.

Cells were cultured for 6 weeks in the presence of irradiated feeders. Each dot represents the concentration of IgM in the culture fluid of a single microwell, 6 weeks after the onset of culture. The dots and vertical lines represent the mean concentration and SD for each group. The dotted horizontal line represents the upper limit of the ELISA.

### 4.2.3.2. The antibody specificity of CD5+ and CD5- EBV lines.

The (auto)specificity of the IgM secreted by CD5+ and CD5- lines was examined by binding to Fc and ssDNA using an ELISA (as per *Materials and Methods* ). Fig.4.6 shows the O.D. values obtained.

Of 120 CD5- lines tested, 72 bound to Fc of IgG with a mean absorbance of 0.52 ±0.42 and 98 to ssDNA with a mean absorbance of 0.38 ± 0.29.

This is compared with the CD5+ lines of which 78 were tested, 29 of which bound to the Fc of IgG with a mean O.D. of 0.46 ± 0.43 and 39 bound to ssDNA with a mean O.D. of 0.47 ± 0.44.

Therefore, there were no significant differences between the CD5+ and CD5- fractionated lines, in that they both produced autoreactive antibodies.

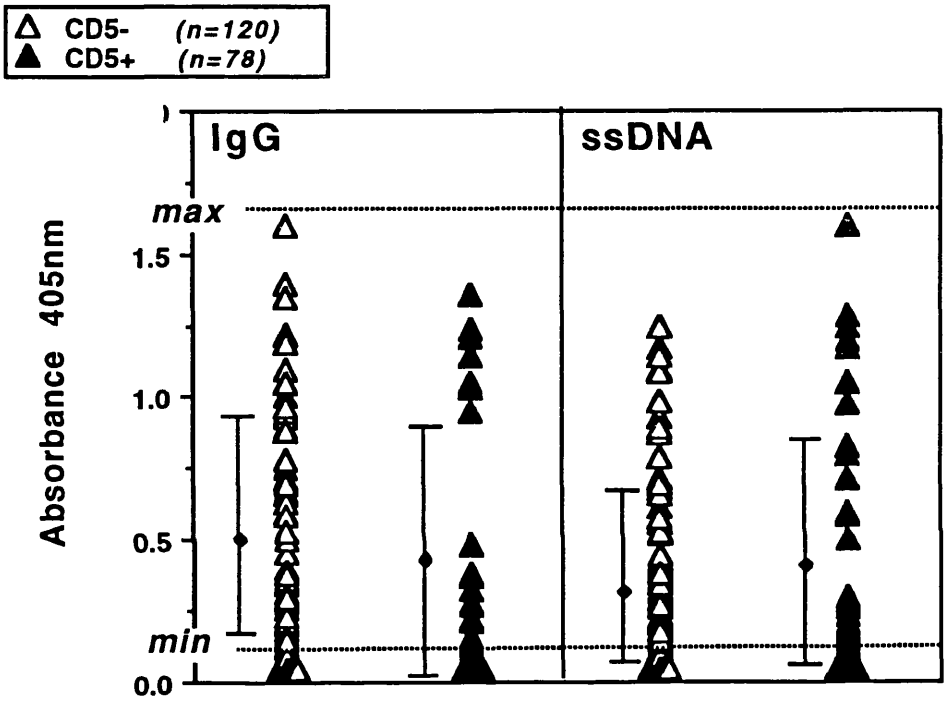


**FIGURE 4.6 ANTIBODY SPECIFICITIES OF CD5+ AND CD5- CORD BLOOD EBV B CELL LINES.** Antibodies produced by CD5+ and CD5- fractionated lymphocytes. Microcultures were plated at 100 cells/well in the presence of 2x $10^4$ irradiated feeders. After 6 weeks, culture fluids from 120 CD5- and 78 CD5+ derived lines, were tested for antigen binding activity to IgG and ssDNA.

Each dot represents the concentration (expressed as absorbance at 405nm) of antibody with a given antigen binding activity in the culture fluid from a single microwell. The dots and vertical lines represent the mean optical density and SD for each group. The dotted horizontal lines represent the limits of the assay, as described in *Materials and Methods*.

## 4.2.4. FURTHER CHARACTERISATION OF CD5+ AND CD5- B CELLS USING *RESTRICTED* CORD BLOOD LINES.

In order to determine whether the multiple specificities, in the cell line supernatants, were due to the presence of more than one reactive clone, we examined individual putative clones for their reactivity to auto-antigens.

### 4.2.4.1. Limiting dilution analysis of CD5+ and CD5- cord blood B cells.

Having observed a higher mean secretion of IgM belonging to the CD5- fraction of sorted cord blood cells (4.2.3.2) we initially, utilising EBV as a polyclonal stimulator, studied the extent of the CD5+ and CD5- precursor population within the cord blood B cells.

This was performed using separated populations obtained from 2 cord blood samples, sorted at different times.

Microtitre plates, each of 96 cultures and Terasaki plates, each of 60 cultures were inoculated, in quadruplet, with graded numbers of B cells derived from CD5+ and CD5- sorted cord blood lymphocytes (0.2, 1,5 and 25 cells/culture) and irradiated feeders.

Fig.4.7a and b shows that in both types of culture plates, CD5- cells display a higher plating efficiency than CD5+ cells in long term culture.

Fig.4.8 a, b, c and d show the immortalization rates of proliferating CD5+ and CD5- B lymphocytes by EBV.

After 6 weeks culture as described, the frequency of immortalization was calculated using Poisson distribution analysis (dotted lines on Fig.4.8 show the cell dose at which

37% of the cultures were negative for transformation) and the inverse of which, determines the frequency of EBV responsive lymphocytes in the CD5+ and CD5- derived populations, Table 4.3, (as described in *Materials and Methods.*).

The data from 2 separate sorts shows that there is a higher number of precursor cells in the CD5- population (0.065±0.006, compared with 0.017± 0.002 in the CD5+ population and 0.087 ± 0.02 compared with 0.021 ±0.001, when the cells are grown in micro or Terasaki culture plates respectively). That is, the CD5- population was 4-5x higher, in terms of responsiveness to the EBV.

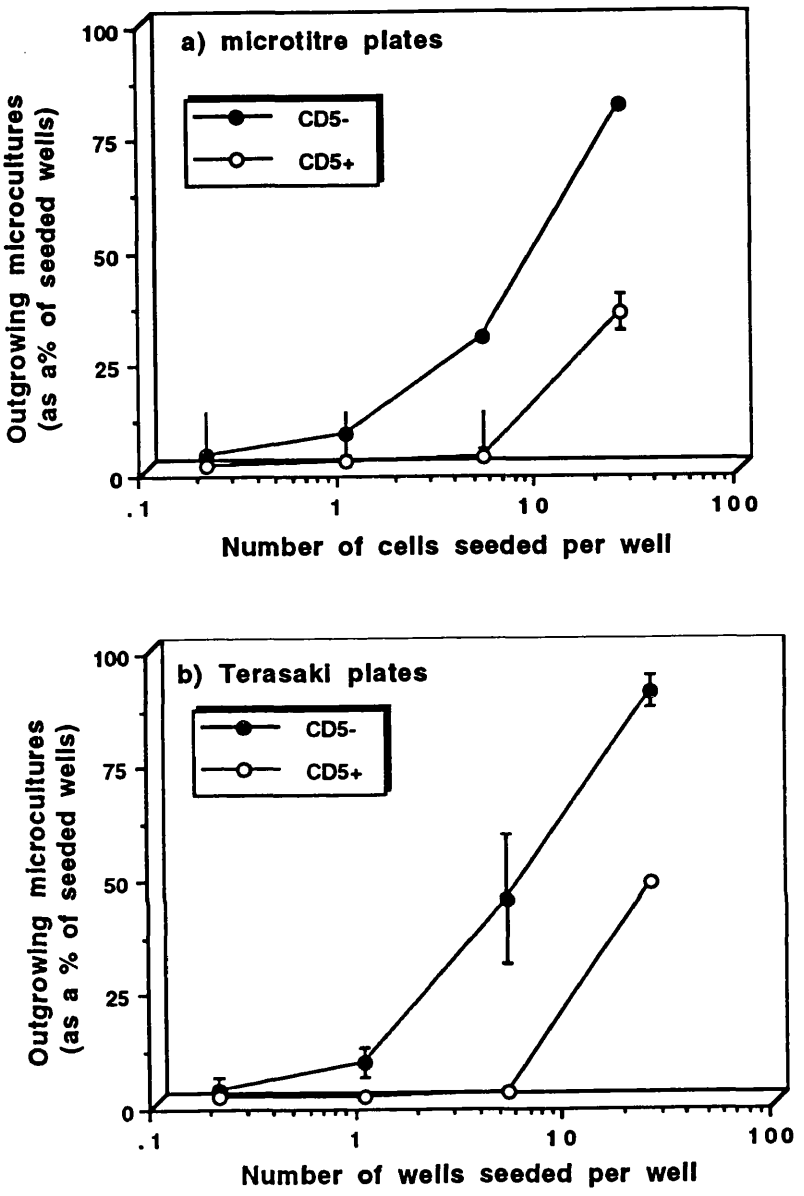


**FIGURE 4.7 GROWTH CURVES FOR EBV INFECTED CD5+ AND CD5- RESTRICTED CORD BLOOD LINES.** The cloning efficiency of EBV infected CD5+ and CD5- fractionated cord blood lymphocytes. EBV transformed B cells were seeded at 0.2, 1, 5 and 25 cells/well into microplates (Fig.4.7a) and Terasaki plates (Fig.4.7b), each seeding carried out in quadruplate plates. All wells contained $2x10^4$ irradiated feeder cells. The mean number of outgrowing wells for each seeding was determined 6 weeks later. Values are expressed as mean and SD (vertical bars) of the percentage of outgrowing cultures, from 2 separate cord blood samples that had been sorted.

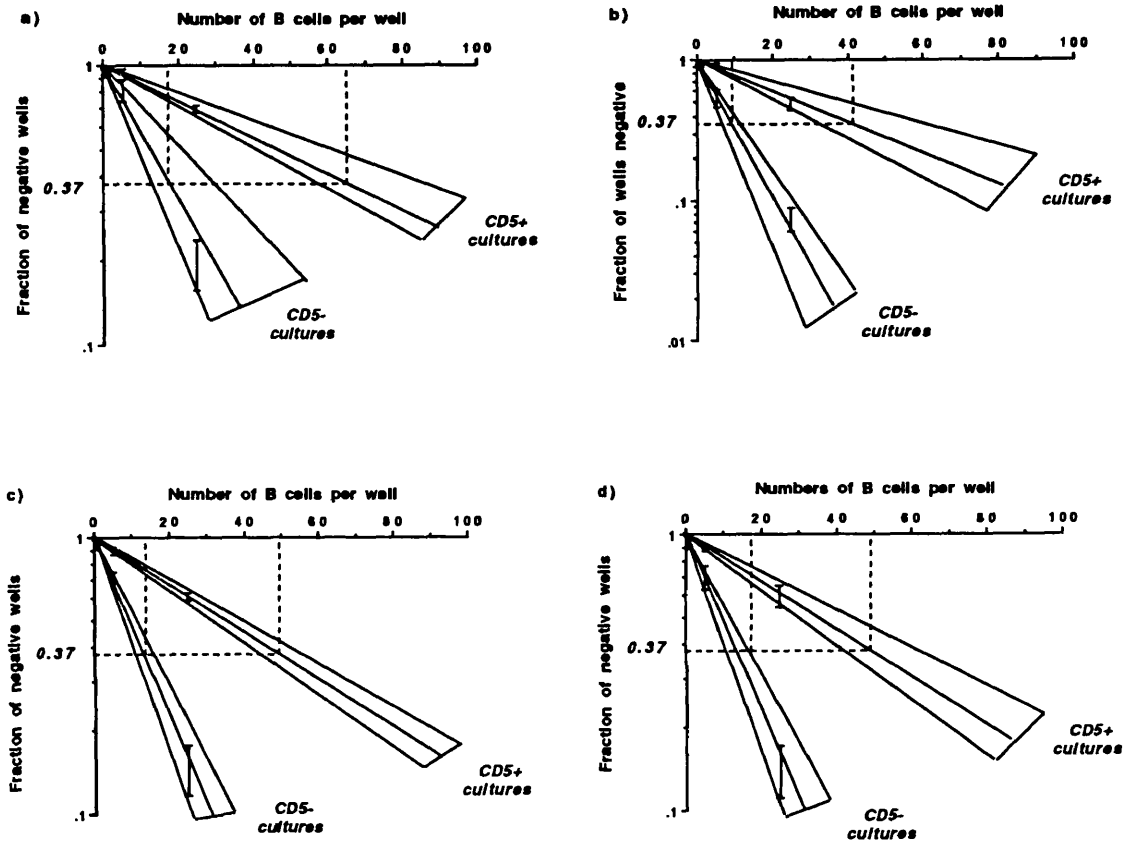


**FIGURE 4.8 ESTIMATION OF PRECURSOR FREQUENCY OF EBV REACTIVE CD5+ AND CD5- CORD BLOOD B CELLS BY LIMITING DILUTION ANALYSIS.** After 6 weeks of culture of 2 separately sorted CD5+ and CD5- cord blood B cells, at different cell seedings, in microplates (Fig.4.8a and c) and Terasaki plates (Fig.4.8b and d), the fraction of microcultures negative for cell transformation, of the total cultures originally seeded, was plotted against cell dose. The frequency of immortalization was calculated as the inverse of the number of cells at which 37% of the cultures were non-responding (indicated by dotted lines). The vertical bars represent the SD for each group. See *Materials and Methods.*

**TABLE 4.3. Precursor frequency of EBV reactive CD5+ and CD5- B cells, from 2 individually sorted cord blood samples : estimated by limiting dilution analysis.**

| Source of cells | CD5+ | | CD5- | |
|---|---|---|---|---|
| | *Microtitre culture plates* | *Terasaki culture plates* | *Microtitre culture plates* | *Terasaki culture plates.* |
| SORT A | 0.015 | 0.023 | 0.058 | 0.111 |
| SORT B | 0.02 | 0.02 | 0.071 | 0.062 |
| Mean *+/- SD* | 0.017 *+/-0.002* | 0.021 *+/-0.001* | 0.065 *+/-0.006* | 0.087 *+/-0.02* |

CD5+ and CD5- derived B lymphocytes infected with EBV. Data are mean values± SD of precursor cell frequencies of EBV responsive cells, cultured in micro and Terasaki culture plates. Description of calculations; see *Materials and Methods*.

All microplate and Terasaki plate wells that were established with growing cells, as described above, from the different cell seedings of EBV infected CD5+ and CD5- lymphocytes, were used in the following studies.

The supernatants collected from all the wells, positive for growth, contained detectable amounts of IgM (data not shown) and all supernatants used in further analysis contained ≥200ng/ml IgM and the majority contained ≥5μg/ml (data not shown).

*Criteria for clonality of B cell cultures :*

In some plates <33% of the wells were positive for growth and IgM secretion. All the supernatants were collected from those wells and assayed for their light chain restriction, by ELISA, to determine clonality.

Expression of only one light chain isotype, together with the Poisson statistic for limiting dilution (Taswell et al, 1981), indicated that these supernatants contained monoclonal IgM and we referred to them as "clones". These clones were selected and expanded in culture for use in the following studies (see *Materials and Methods* )

**4.2.4.2. Frequency of κ and λ bearing IgM clones derived from the CD5+ and CD5- cord blood B cells.**

**a)** The light chain analysis of the putative clones derived from both the CD5+ and CD5- cord B cells is shown in Fig.4.9.

Significantly higher frequencies of kappa clones were noted in the CD5+ population (54/86), compared with the CD5- poulation (30/75), ($p<0.01$). κ : λ ratios were 1.7 : 1 and 0.7 : 1 respectively.

**b)** Since we were concerned that EBV could be specifically immortalizing a subpopulation of B cells, we analysed the percentage of B cells that expressed kappa and lambda light chains in native, unseparated cord blood, from 9 donors.

There were no significant differences between the frequencies of kappa and lambda bearing cells, in the native, unseparated group, compared with the frequencies of kappa and lambda bearing cells, in the panel of 161 EBV cord blood clones in the study.

$\kappa : \lambda$ ratios were 1.02 : 1 and 1.09 : 1, respectively (Fig.4.10).

These two groups were found to contain comparable numbers of CD5 B cells : 53% of the EBV cord blood clones analysed, were derived from CD5+ B cells and analysis of the 9 cord blood samples used showed that the percentage of B cells expressing surface CD5 was 48%±16.

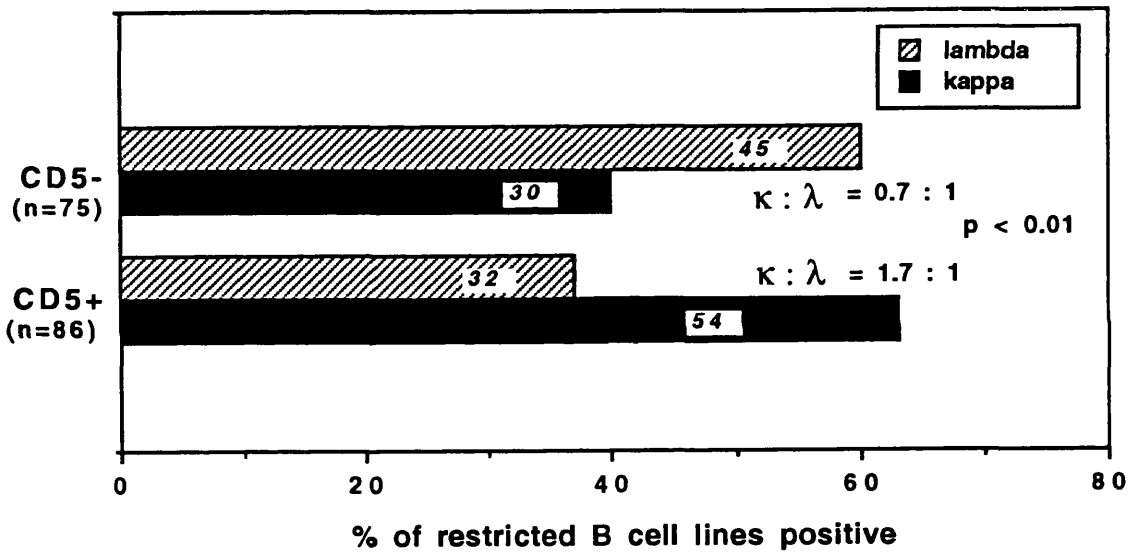


**FIGURE 4.9 LIGHT CHAIN EXPRESSION OF CD5+ AND CD5- DERIVED CORD BLOOD EBV B CELL CLONES.** The frequency of kappa and lambda light chain expression by 86 CD5+ and 75 CD5- derived cord blood B cell restricted lines. Results are expressed as the percentage positive, as determined by ELISA. The relative ratios of $\kappa$ to $\lambda$ light chains are shown. The p value is the result of a comparison of B cells expressing $\kappa$ or $\lambda$ in the CD5+ with the CD5- groups.

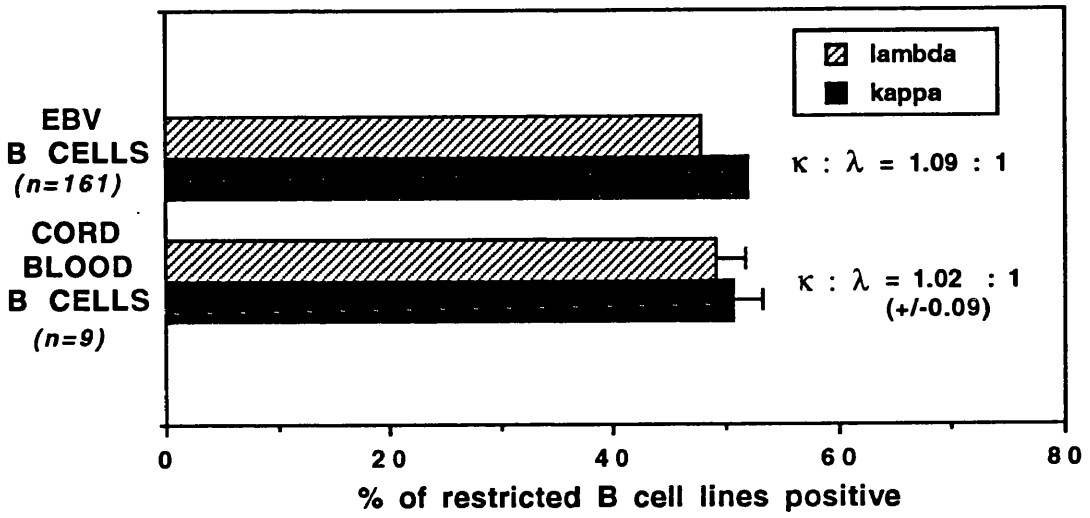


**FIGURE 4.10 RATIOS OF KAPPA : LAMBDA (κ:λ) LIGHT CHAIN EXPRESSING B CELLS IN UNSTIMULATED COMPARED WITH EBV STIMULATED CORD BLOOD B CELLS.** The frequency of kappa (κ) and lambda (λ) light chain bearing B cells from 9 cord blood donors and 161 EBV transformed cord blood restricted lines. Results are expressed as the percentage of cells found to be positive by ELISA and by immunofluorescence and flow cytometric analysis. The ratios of κ to λ B cells are indicated. The horizontal bars represent the SD for the mean values of the donor cord blood B cells.

### 4.2.4.3. Morphology and phenotype of cord blood EBV clones

During the course of these studies, the cord blood EBV clones, under examination, were in continuous cell culture.

It was observed that there were striking differences in the macroscopic patterns of growth of the individual EBV B cell clones, cultured during the course of these investigations.

Many cultures grew, at all times, as small groups or even as individual cells in suspension, while others remained as extremely large clumps of cells (Fig.4.11).

Viewing by eye or with the aid of an inverted microscope, no correlation could be made between these features of growth and CD5 positivity, as both CD5+ and CD5- derived clones displayed them. Fig.4.11 a-d shows representative examples of the types of cultures observed. Cells were seeded at $10^6$/ml, 72 hours prior to being photographed.

A small phenotypic study, revealed no correlation in the morphological features of the cells and the expression of surface molecules, CD5 (UCHT2), CD23, CD21 or the adhesion molecules, ICAM-1 (84H10) and LFA-1 (TMD3-1), kindly provided by Prof. PCL Beverley, ICRF, London (data not shown)

None of the clones analysed in this small study expressed the CD5 molecule, which was the case for 92% of 161 clones which were examined for the surface expression of CD5 (data not shown).

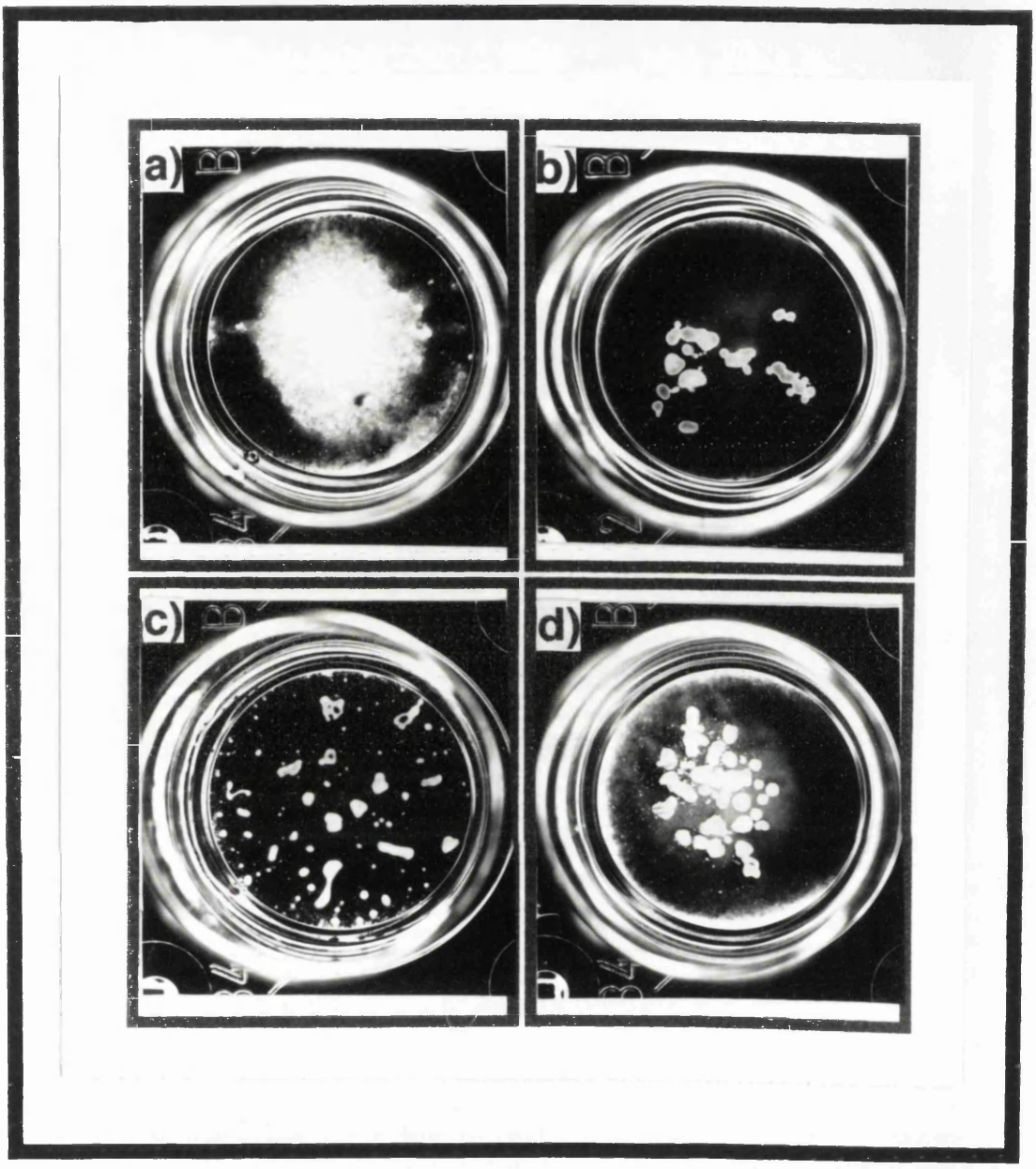


**FIG 4.11 EXAMPLES OF MORPHOLOGY OF CULTURES OF CD5+ AND CD5- EBV CORD BLOOD CLONES.** Representative patterns of growth of 2 CD5+ (a and b, clones 236 and 169 respectively) and 2 CD5- (c and d, clones 116 and 78 respectively).

Cells were seeded at $10^6$ cells/ml in complete culture medium (see *Materials and Methods* ) and photographed after 72 hours.

**4.2.4.4. Binding of IgM from CD5+ and CD5- clones to a panel of autoantigens.**

On the basis that they satisfied our criteria for clonality, described above (and see Materials and Methods), 86 CD5+ and 75 CD5- cord blood EBV B cell *clones* were examined in the following studies.

Table 4.4 shows that both CD5+ and CD5- cord blood clones are autoreactive and polyreactive. The relative numbers of clones with specificities for the different autoantigens of the total were examined.

A high frequency of clones reacted with IgG, ssDNA and type I/II collagen (eg. 34κ and 10λ out of 86 CD5+ clones). Many were also polyreactive, as defined by reactivity with two or more unrelated antigens (eg. IgG and ssDNA). Both CD5+ and CD5- clones reacted with one or more autoantigen but clones derived from the CD5- B cells had a significantly lower frequency of this activity (Table 4.4) :

i. 50% (43/86) of the CD5+ clones compared with 28% (21/75) of the CD5- clones produced rheumatoid factor (RF), ($p<0.01$) and 51% (44/86) of CD5+ clones and 24% (18/75) of the CD5- clones produced anti-ssDNA antibodies ($p<0.001$).

ii. Specificity directed towards ssDNA was significantly more frequent in the kappa (34/54) than in the lambda (10/32) clones within the CD5+ group ($p<0.01$), as was multispecificity, directed towards Fc of IgG (RF) and ssDNA, (26/54 κ compared with 7/32 λ clones ; $p<0.02$).

**TABLE 4.4 Summary of the antigen specificities of the CD5+ and CD5- cord blood EBV clones.**

| CORD BLOOD CLONE | SPECIFICITY | | | | | |
|---|---|---|---|---|---|---|
| | RF | ssDNA | RF | CoIII/II + ssDNA | ssDNA | ALL + CoIII/II |
| CD5+ * | | | | | | |
| κ | 31 | 34 | 26 | 15 | 15 | 15 |
| λ | 12 | 10 | 7 | 6 | 5 | 5 |
| | | $p < 0.01$ | $p < 0.02$ | | | |
| CD5- ** | | | | | | |
| κ | 9 | 8 | 7 | 4 | 3 | 3 |
| λ | 12 | 10 | 7 | 6 | 5 | 5 |
| | $p < 0.01$ | $p < 0.001$ | | | | |

* IgM supernatants from 54 kappa and 32 lambda CD5+ and ** 30 kappa and 45 lambda CD5- clones were analysed for binding to Fc of IgG (Rheumatoid factor, RF), single-stranded DNA (ssDNA) and Collagen Types I and II (Coll I/II).
The p values are the result of the comparisons of B cells exhibiting specificities within the CD5+ group, between κ and λ clones and the comparisons of the specificities of the CD5+ clones with the CD5- clones.

To further analyse the similarities of the autoantigen specific clones within the CD5+ and CD5- lymphocyte populations, individual specificities of 26 CD5+ and 32 CD5- clones were assayed for a more complete autoantigen panel, shown in Fig. 4.12a and 12b respectively.

IgM from some of both CD5+ and CD5- B cell clones had specificity to all the antigens in the panel and the

majority to more than one, confirming the polyreactive nature of many of the clones.

Interestingly, higher numbers, though not significantly, of CD5- clones (17/32) displayed specificity for mitochondrial antigens, when compared to the CD5+ clones (1/26). This was particularly apparent in the lambda clones, (12/19 CD5- λ compared with 0/5 CD5+ λ).

A few clones reacted with other antigens, by immunofluorescence, not shown on the table, for example, #199 reacted with the golgi apparatus and #106 , 169 and 189 displayed anti-nucleolar specificities.

Some of the binding specificity patterns are shown in Fig. 4.13a, b, c and d.

No clones produced IgM which bound to thyroglobulin using the coated erythrocyte indicator system in this study.

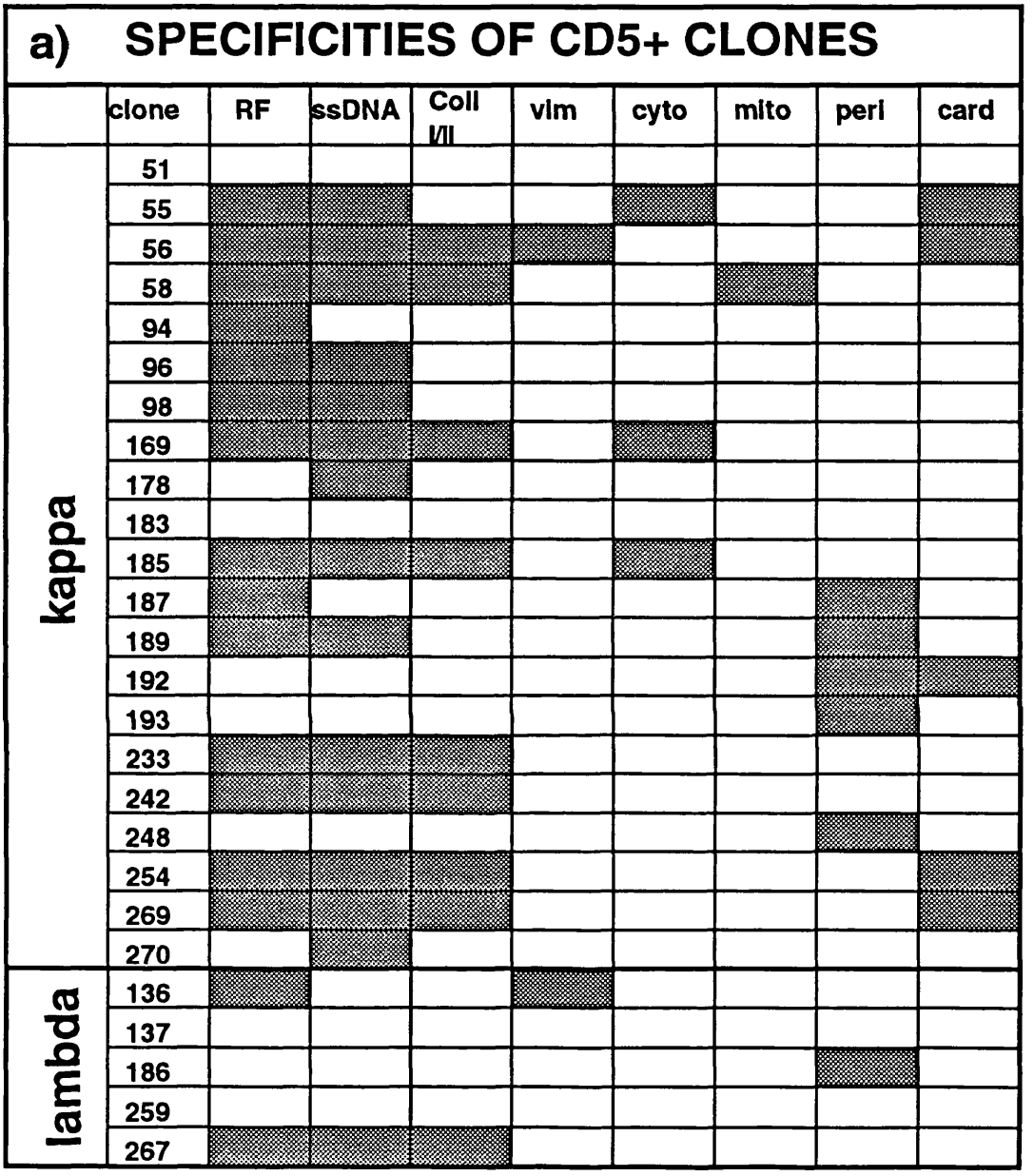


**FIGURE 4.12a FURTHER SPECIFICITIES OF CD5+ CORD BLOOD EBV CLONES.**

**KEY** : RF, rheumatoid factor, ssDNA, single-stranded DNA, coll I/II, collagen types I and II, vim, vimentin, cyto, cytokeratin, mito, mitochondria, peri, perinuclear, card, cardiolipin.

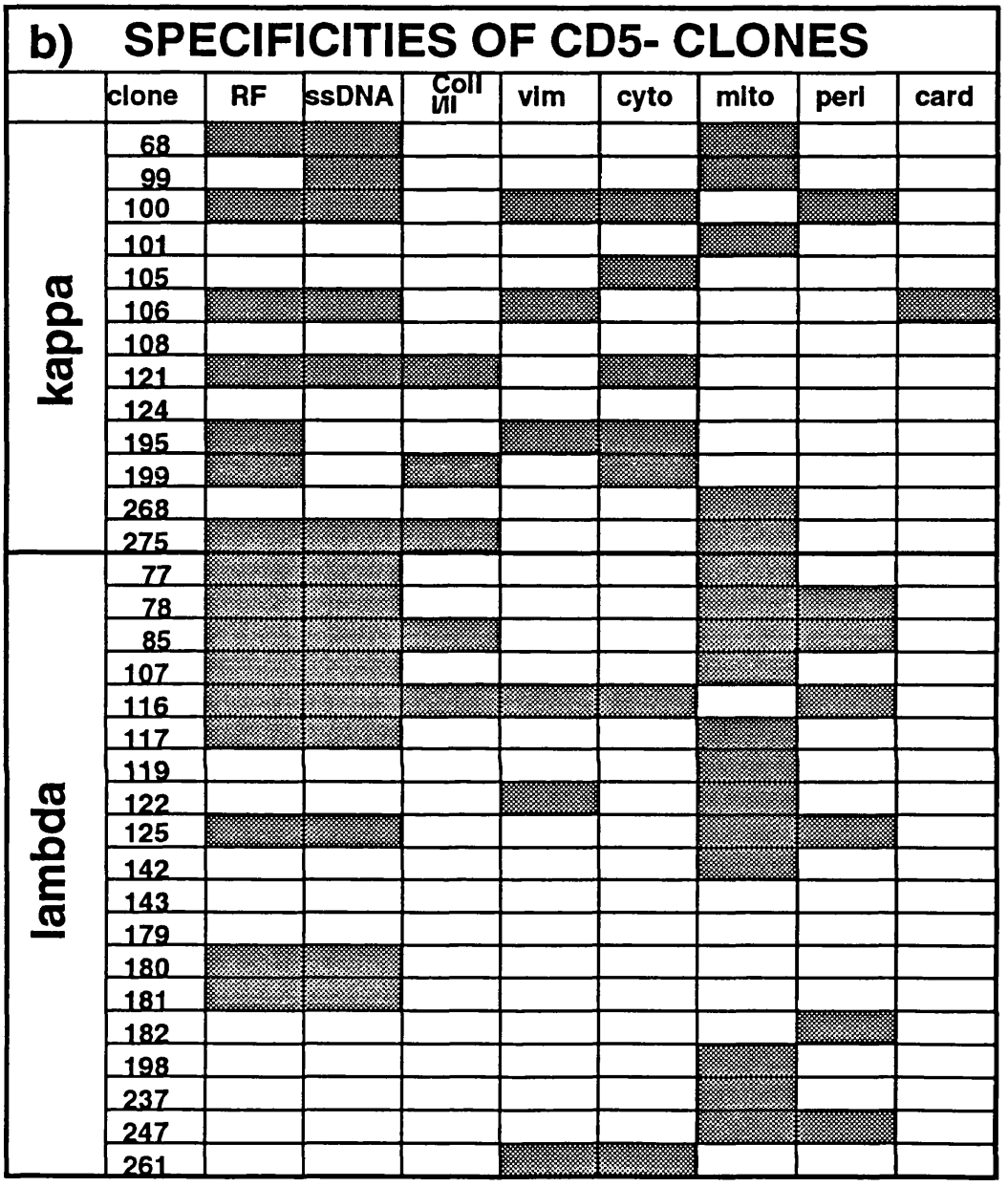


**FIGURE 4.12b FURTHER SPECIFICITIES OF CD5- CORD BLOOD EBV CLONES.**

**KEY** : RF, rheumatoid factor, ssDNA, single-stranded DNA, coll I/II, collagen types I and II, vim, vimentin, cyto, cytokeratin, mito, mitochondria, peri, perinuclear, card, cardiolipin.

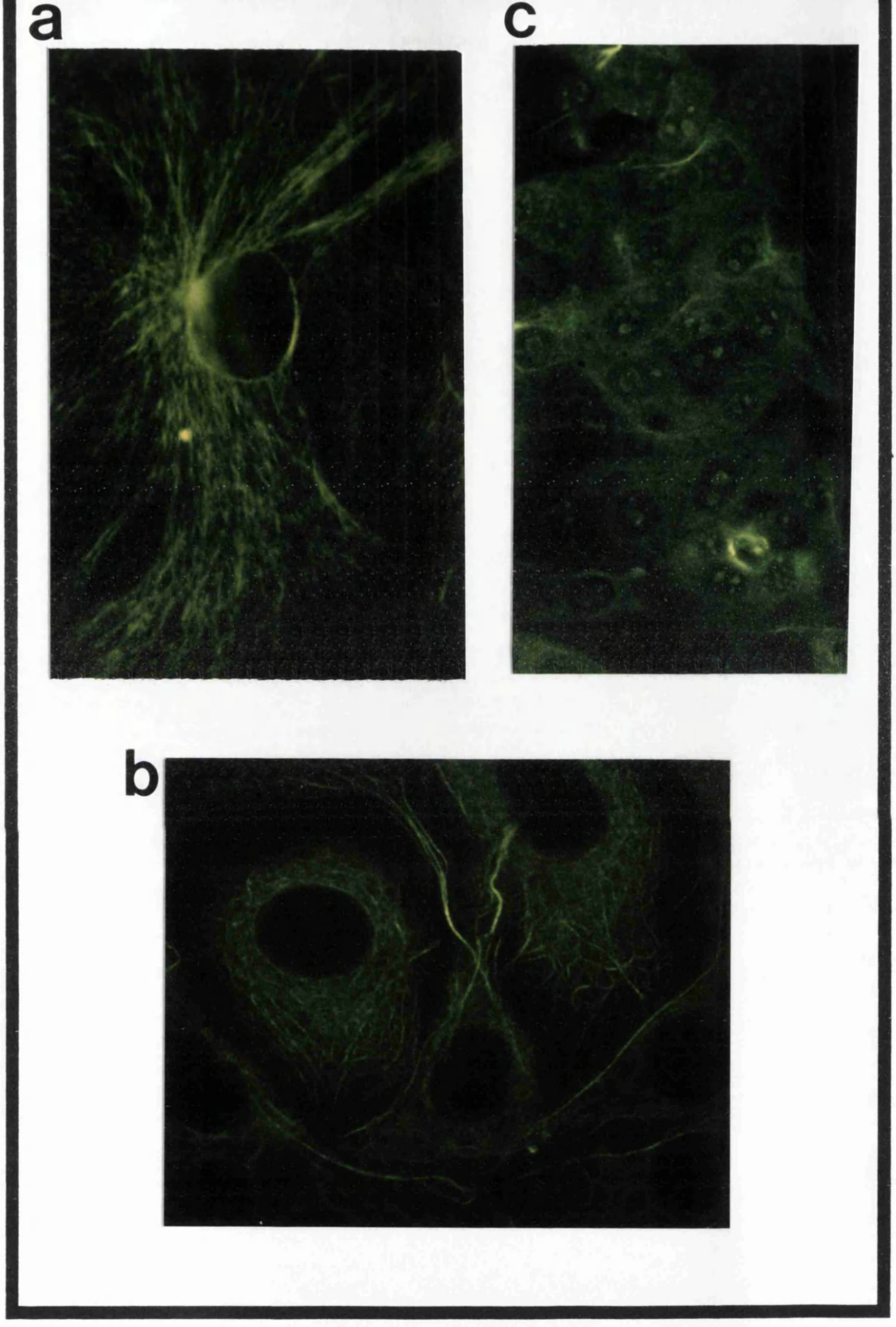


**FIGURE 4.13 ANTIBODIES TO CYTOSKELETAL COMPONENTS.** Examples of anti-vimentin (a), anti-cytokeratin (b) and anti-nuclear with anti-nucleolar staining (c), by cord blood clone supernatants. Cells of the IMR-33 fibrosarcoma line (a and b) and Hep 2 cells (c) were stained, as indicated in Materials and Methods.

## 4.2.5. AVIDITY OF IgM FROM CD5+ AND CD5- CORD BLOOD CLONES.

Our findings had clearly established that IgM collected from the panel of both CD5+ and CD5- EBV transformed cord blood clones, can be autoreactive and polyreactive.

To formally prove the antigen binding activity of these monoclonal autoantibodies and to further investigate their properties, we performed dose-dependent binding assays, using IgM from both groups, to different self-molecules (IgG Fc, ssDNA and collagen Types I and II).

Further, as an approach to studies of avidity and to confirm the polyreactivity of the antibodies, we then examined the IgM in inhibition assays, in which the binding of a given monoclonal antibody to a solid-phase antigen, was tested in the presence of soluble antigen.

### 4.2.5.1. Binding curves of IgM from CD5+ and CD5- clones to autoantigens.

Monoclonal purified IgM (see *Materials and Methods*), from both CD5+ and CD5- cord blood B cells bound to IgG, ssDNA and Collagen type I/II in a dose dependent fashion with similar binding curves.

The antigen binding curves of representative polyreactive monoclonal antibodies, generated from a CD5+, kappa clone, designated clone 169, and generated from a CD5-, kappa clone, designated clone 121, are shown in Fig.4.14.

IgM from 6 CD5+ and 7CD5- cord blood clones was examined and yielded comparable results (data not shown), ie, all of these polyreactive monoclonal antibodies, bound to two or more of Fc fragment of IgG, single-stranded DNA and

collagen Types I/II, in a dose-dependent fashion. Interestingly, as illustrated in Fig 4.14, binding slopes with different shapes, were derived, when similar concentrations of the same antibody were tested on different antigens.

Moreover, binding slopes with different shapes, were seen when different antibodies bound to the same antigen with different efficiency.

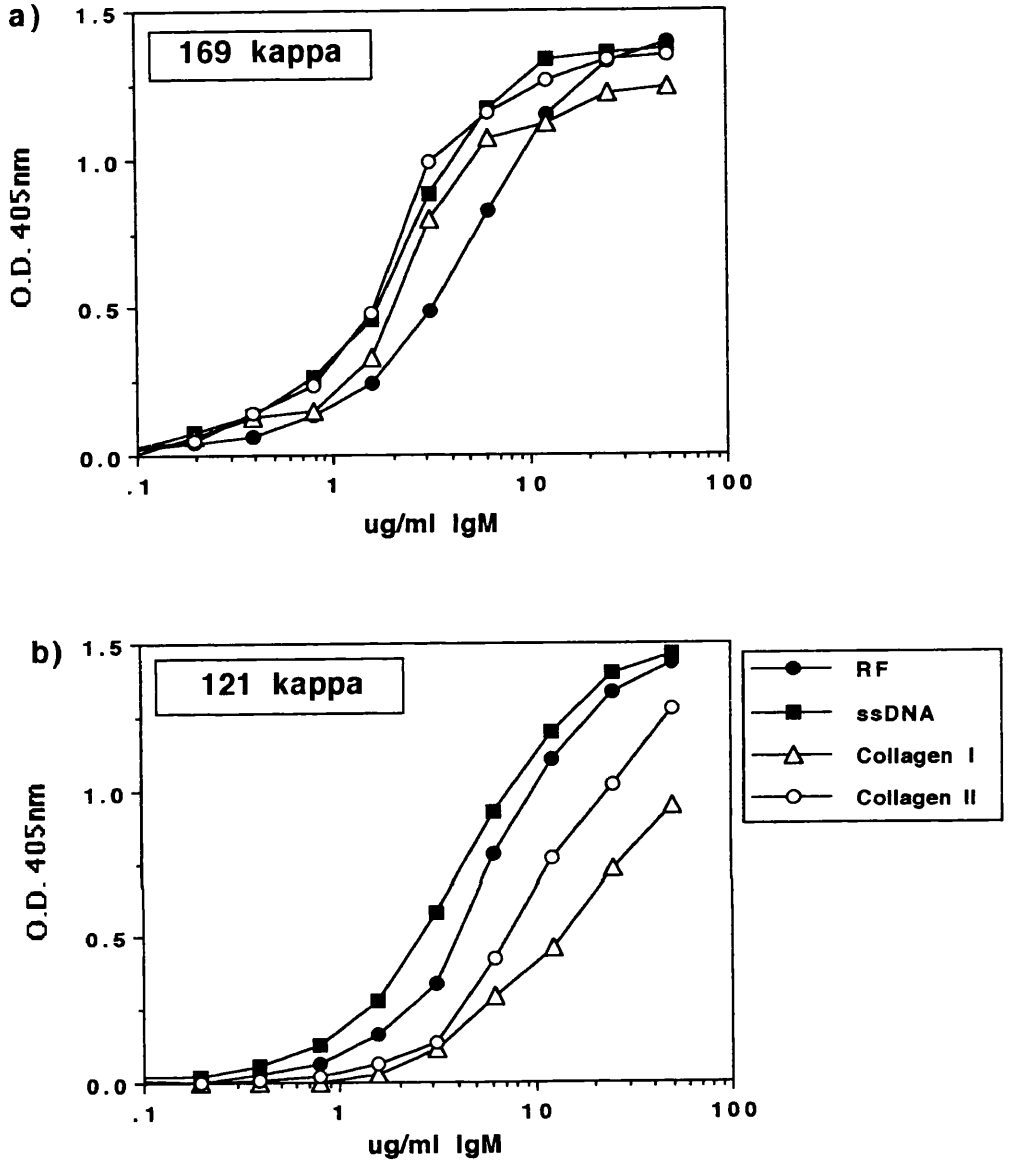


**FIGURE 4.14 BINDING CURVES OF IgM FROM CD5+ AND CD5- CORD BLOOD CLONES TO AUTOANTIGENS.** Dose-dependent binding of monoclonal IgM from CD5+, kappa clone, 169 (Fig.4.14a) and from CD5-, kappa clone, 121 (Fig.4.14b), to solid-phase IgG Fc fragment (Rheumatoid factor, RF), single-stranded DNA (ssDNA) and collagen Types I and II (Coll I/II). The antigen binding activity for each group is expressed as optical absorbance (OD) at 405nm. The threshold for positivity was equivalent to mean +/- x2 standard deviation (SD) of culture fluid which was negative for the antigens tested (generally 0.075). Dilutions of bovine serum albumin (BSA) were also included as a negative control. The antigen binding activity of 13 other polyreactive monoclonal antibodies from CD5+ and CD5- B cells were similar.

**4.2.5.2. Both CD5+ and CD5- clones produce IgM antibodies of low avidity.**

The results above (4.2.5.1.), suggested that the polyreactive IgM, generated from both CD5+ and CD5- cord blood B cells, have similar avidity.

We investigated this further using inhibition studies, in which the binding of a 2.5μg of a given monoclonal antibody to a solid-phase antigen was tested in the presence of increasing amounts of the homologous soluble antigen (see *Materials & Methods*).

The binding of each antibody observed in the presence of a soluble ligand is expressed as a percentage of the binding measured after incubation of the antibody, under identical conditions, but in the absence of any soluble ligand (100% binding activity). In all cases, the amount of unblocked antibody bound was measured as absorbance, (405nm) and was always at least 0.4.

Fig. 4.15 shows that the binding of IgM from CD5+ clone, 169 to IgG, ssDNA and Collagen types I/II, was inhibited in a dose-dependent fashion and with different efficiency by soluble IgG, ssDNA and Collagen I/II, Fig 4.15a. At least 100μg of soluble ligand, was required to inhibit 50% of the antibody binding (indicated by dotted lines, suggesting low avidity.

Although there were variations, within each monoclonal examined, generally, similar results were found when 6 other, polyreactive, CD5+ clones were examined in this way, ( mean value of 122 μg IgG +/- 39 and 163 μg ssDNA +/- 70.3, were required to inhibit binding to solid-phase IgG and ssDNA, respectively, by 50%).

Fig. 4.15b shows that the binding of IgM from CD5-

clone, 121, to IgG, ssDNA and Collagen I/II was also inhibited in a dose dependent fashion and with different efficiency, by soluble IgG, ssDNA and Collagen I/II. In this case also, large amounts of soluble ligand were required to inhibit binding to the solid-phase ligand by 50% (indicated by dotted lines).

Similar data was obtained when 3 other, polyreactive, CD5- clones were examined (mean value of 192.5μg IgG +/- 70.3 and 163.8μg ssDNA +/- 133.2, was required to inhibit binding to solid-phase IgG and ssDNA, respectively, by 50%).

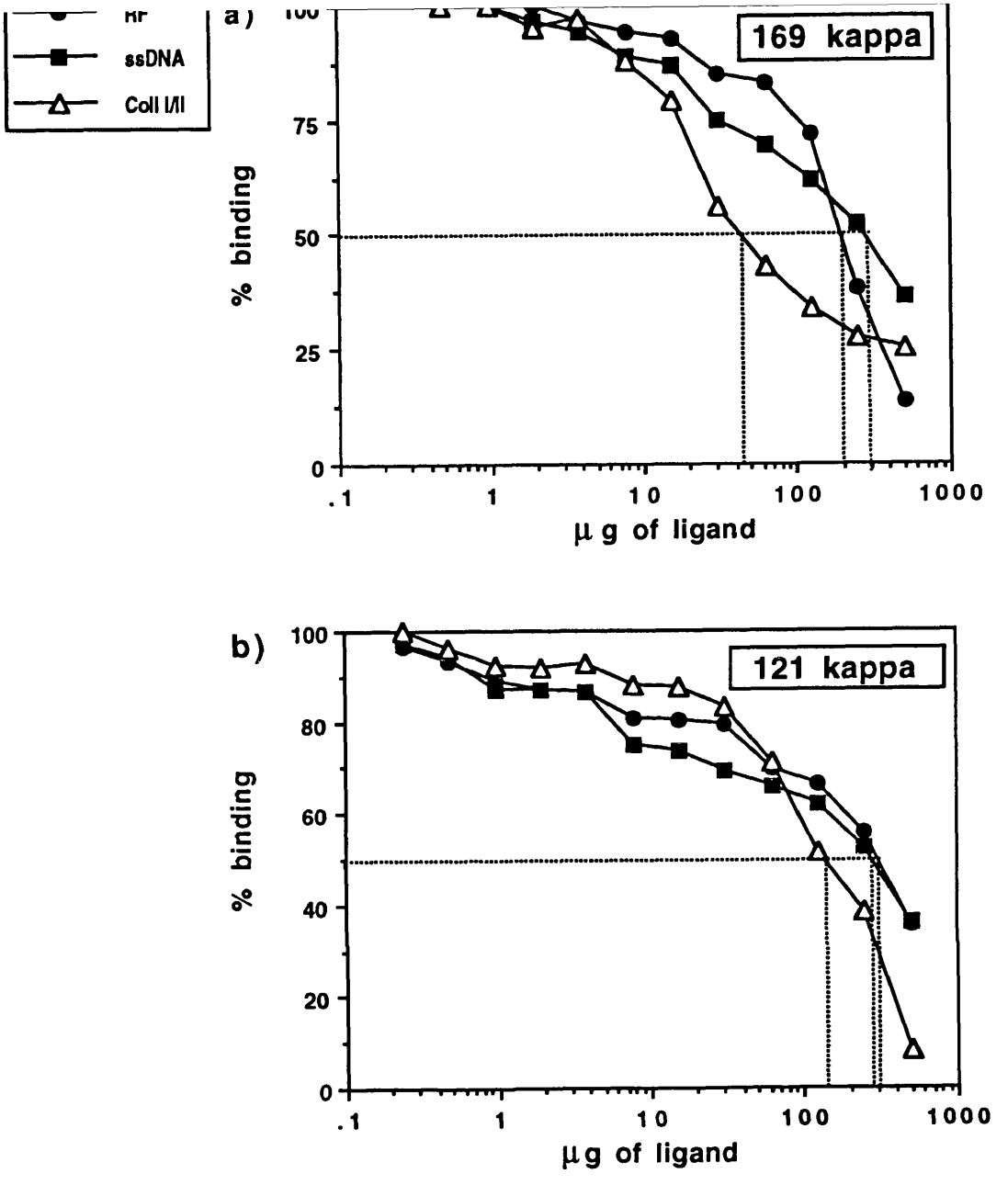


**FIGURE 4.15. INHIBITION OF BINDING OF IgM FROM CD5+ AND CD5- CORD BLOOD B CELL CLONES TO AUTOANTIGENS.** Dose-dependent inhibition of monoclonal IgM from CD5+, kappa clone, 169 (Fig.4.15a) and CD5-, kappa clone, 121 (Fig.4.15b), to solid-phase IgG Fc (Rheumatoid factor, RF), single-stranded DNA (ssDNA), and collagen Types I/II (Coll I/II), by soluble homologous Fc of IgG, ssDNA and collagen I/II, respectively.

The binding of IgM, observed in the presence of soluble ligand, is expressed as a percentage of the binding activity measured after incubation of that IgM under identical conditions, but in the absence of any soluble ligand (100% of binding activity) (see Materials and Methods). The 100% binding activity in each case corresponded to an Optical absorbance (OD) of at least 0.4 at 405nm. Dotted lines indicate the amount of soluble ligand required to inhibit binding by 50%.

## 4.3 DISCUSSION

In this study, we investigated the relationship between CD5 positivity and autoantibody producing B cells.

### 1. Specificity of CD5+ chronic lymphocytic leukemic B cells.

The antibody producing potential of chronic lymphocytic leukaemia, (CLL) B cells, was investigated since the majority of the malignant clones express CD5. This confirmed other reports, (Wang et al, 1980).

Most B-CLL clones closely resemble mature, resting B lymphocytes (Fu et al, 1974, Salsano et al, 1974 and Seligmann et al, 1974), although it had been reported that the proliferative response of B CLL to mitogens was generally very poor, and differentiation only induced in exceptional cases (Godal and Funderud, 1982). However, phorbol ester has been described to be a potent inducer of differentiation in about 60% of B CLL clones investigated, the majority, developing a lymphoblastoid/plasmacytoid morphology and becoming secretors of IgM (Totterman et al, 1980). In these studies, using PMA as a differentiation agent (Forsbeck et al, 1987 and Larsson et al, 1987), peripheral blood mononuclear cells, in the majority of the CLL patients examined, were induced to secrete considerable amounts (>200ng/ml) of IgM. That the IgM collected, originated from the malignant clones, was confirmed by light chain analysis.

Examining the reactivity of the antibodies produced, revealed a high proportion of CLL B cells to be autoreactive, with specificity for the Fc of IgG, in particular. This is consistent with previous reports on various paraproteins

(Metzger et al, 1969, Kunkel et al, 1973, Dighiero et al, 1983, Shoenfeld et al, 1986 and Seligman and Brouet, 1973 and 1984). Further studies, performed by Dr. B. Broeker, in our laboratory, revealed that the same IgM supernatants could be concentrated and shown to be both autoreactive and polyreactive (Broeker et al, 1990).

Autoimmune disease is occasionaly to be found in patients with CLL and interestingly, these diseases are more frequent in relatives of patients with CLL, than in the general population (Hamblin et al, 1986).

It is a novel idea to propose the involvememt of a common genetic factor in both autoimmune disease and the development of malignancy (Conley et al, 1980). CD5 B cells, which are part of the normal B cell pool have a proposed involvement in the generation of autoantibodies (Casali et al, 1987 and Hardy et al, 1987).

The data presented here, demonstrates at the cellular level, that CD5 positive B CLL cells may secrete autoantibodies and therefore may even represent immortalized clones of these autoreactive B cells. Furthermore, it's tempting to speculate that the proliferative capacity of CD5 B cells (Hardy and Hayakawa, 1986) and their stimulation by antigen, may contribute to the malignant B cell transformation, frequently seen in patients with autoimmune disease.

Some of the features of B CLL may be aberrantly expressed as a consequence of the malignant transformation. It may therefore, be difficult to postulate the exact phenotype of the normal CD5 B cell counterpart of CLL. However, in some recent, elegant experiments by Kipps et al, 1990)

evidence has been given for a selection of specific V genes used by CLL patients, suggesting that they might indeed represent a malignant clone of CD5 B cells, selected for their antigen specificity.

## 2. Probing the human CD5 B cell repertoire with Epstein-Barr virus (EBV).

Epstein-Barr virus (EBV) was employed to stimulate neonatal B cells, rich in CD5 B cells, to immunoglobulin secretion. The unique advantage of EBV, is that it serves as both a potent B cell activating stimulus and an efficient transforming agent.

Initially, cord blood and fetal liver B cells were activated in bulk cultures and it was shown that a large percentage of B cell lines established, produced IgM autoantibodies. Since EBV has been shown to bind, with equal efficiency, to cells bearing immunoglobulin heavy chains of all isotypes (Inghirami et al, 1988), the predominant IgM production found, throughout all these experiments, simply reflected the higher frequency of IgM programmed lymphocytes in the cord blood B cell repertoire.

Interesting experiments, using a culture system similar to the one described here (Chan et al, 1986), show that a major proportion of IgM committed EBV transformable B cells are derived from a population of large, activated lymphocytes. In contrast, precursors of IgG or IgA producing lymphoblastoid cell lines, are small resting lymphocytes. An extension of these findings to the interpretation of my data, would imply that the antigen reactivity of the EBV transformable cells described, reflects a population of

"activated" B cells. Whether this is a reflection of the nature of the CD5+ B cell subpopulation, could not be realised without sorting these cord blood B cell into isolated CD5+ and CD5- subpopulations.

My studies demonstrated that, CD5+ cord blood B cells, can be separated as a discrete cell subset from CD5- B lymphocytes, by fluorescent activated cell sorting (FACS) and that by infection with EBV, both subpopulations can be induced to secrete immunoglobulin molecules of the IgM isotype.

Interestingly, although the majority, of both the CD5+ and CD5- cells, in cultures seeded at 100 cell/culture, secreted high concentrations of IgM (generally at least 10$\mu$g/ml), significantly higher concentrations were secreted by the CD5- cultures. However, the frequency of EBV activated B cell cultures with antigen specificity, as determined by expressing them as a fraction of the total number of IgM producing cultures (data not shown), revealed no significant differences between the CD5+ and CD5- derived cell lines.

Studies of such restricted lines, derived from these separated CD5+ and CD5- cells, revealed that both populations of B cells can be induced to produce autoantibodies. These antibodies can bind to autoantigens such as Fc of IgG, ssDNA collagen, typeI/II and various cytoskeletal components. However, since there were many different cells present, it was not possible to ask the question, whether both CD5+ and CD5- B cells produce polyreactive antibodies. Furthermore, it seems likely that cognitive interactions between B cells in such bulk cultures, may positively or negatively influence the fate of individual members. Finally, it is interesting that CD5

B cells from different, individual aged NZB mice often show "all or none" reactivity to Br-MRBC, depending upon which clone has been selected (Ansar et al, 1989). Therefore, changes in the environment will change the proportion of secreting cells within a population of B cells with the same specificity.

Although EBV transformed B cells are a useful source of monoclonal antibodies, I found that they can be cloned only with difficulty at low cell numbers (<5 cells/well) and their growth and antibody production are erratic when cultured beyond a short period. However, I managed to establish and maintain clones of B cells derived from these cultures, as defined on the basis of limiting dilution analysis and light chain restriction (see *Materials and Methods*).

Under limiting dilution conditions, in all situations, despite varying culture environments, ie growing B cells with/without feeder cells, the frequency of EBV responsive lymphocytes was higher (up to x5) in the CD5- population. This pattern of growth confirmed the earlier observation that CD5- derived lines secreted a higher concentration of IgM into the culture supernatants, when compared with the CD5+ derived lines.

The apparent lower cloning efficiency of B cells reponding to EBV in the CD5+ population may reflect a state of stimulation within this subpopulation, that is different to that of the CD5- subpopulation.

It may simply be that pertubation of the cell membrane by attachment of anti-CD5 monoclonals prior to sorting, was sufficient to affect their ensueing survival and

growth in culture, or indeed, their response to EBV transformation. T cells can be activated by cross-linking of CD5 antibodies coated onto solid-phase (Ceuppens and Baroja, 1986) and this signal alone is required for T cell proliferation (MacKenzie et al, 1989). Indeed, CD72, the ligand for CD5 (van der Velde et al, 1991), likely enhances B-B cognate interactions and may well enhance activation.

Interestingly, it has been established that CD5 antibodies augment the production of RF by human lymphocytes *in vitro* (Hara et al, 1988). This is, however, more likely to be through an indirect action by T cells.

Finally, since both CD5+ and CD5- subsets of B cells *do* respond to EBV, both must contain B cells, early in their differentiation pathway (Silverman et al, 1987) and in the early stages of B cell activation (Crain et al, 1989). However, the lower precursor frequency observed for CD5 B cells, responding to EBV, maybe argues against EBV CD5 B cells being activated. Therefore, the possibility that there is a preferential selection by EBV, of IgM producing, autoreactive clones, in this experimental system, remains.

Since B cells in a terminal differentiation stage, lose surface CR2 (Boyd et al, 1985), lack of EBV binding to cells in S phase of the cell cycle, or plasma cells, could explain a lower rate of virus induced immortalization. Interestingly, a lower rate of transformation by EBV, is occassionaly observed, *in vivo*, in patients with an active autoimmune disease (Irving et al, 1985).

Whether the population of CD5+ B cells infected with EBV in this study, were already "activated", through stimulation within their internal environment, or are

influenced by our experimental procedures, is unresolved.

The monoclonal antibodies in these studies, induced from both CD5+ and CD5- B cells, proved to display various patterns of reactivity against the panel of autoantigens we had available to us, particularly towards the Fc of IgG and ssDNA. A number were polyreactive, in that they reacted with at least two different autoantigens, while others were more restricted in their nature.

Unfortunately we were unable to ascertain the precursor frequencies of the CD5+ and CD5- B cells responding to the autoantigens in the study. However, the higher frequency of autoreactive CD5+ B cells detected in this study, could indicate that there are more B cells primed against endogenous autoantigens within this subset.

These results suggested that autoantibody V genes are expressed in the preimmune, neonatal human repertoire.

My data is consistent with that obtained from newborn mice, in which hybridomas were generated with specificity for cytoskeletal components and DNA (Dighiero et al, 1985). It is likely, therefore, that the antibodies we isolated in these studies are the "natural" antibodies described by Dighiero et al, 1983. Natural antibodies are produced by normal and tumour B cells and react with a variety of different antigens, both self and non-self and frequently with structurally conserved antigens.

The autospecificities of the isolated CD5+ and CD5- cord blood B cells were to be found in both kappa and lambda light chain expressing clones, indicating that polyreactivity was not a feature of cells expressing one type of light chain.

In this study, the κ:λ ratio was significantly higher in the CD5+ B cells than in the CD5- cells and is consistent with our previous data (Chapter 2), indicating that there is not a bias for λ light chain expression by human CD5 B cells, as there is in the mouse (Hayakawa et al, 1986).

Furthermore, in most cases, the frequency of IgMκ expressing clones, which were auto and polyreactive to the panel of antigens tested, was higher than the λ bearing cells in both the CD5+ and CD5- groups. This was only significant in the CD5+ population when clones producing IgM with specificity for ssDNA were compared.

Selection by EBV was to some extent excluded by evidence that there was no significant differences to be found in the κ:λ ratio when cord blood B cells are compared to the EBV B cell clones in this study, both containing comparable percentages of CD5 B cells.

Other experiments performed in our laboratory (Paavonen et al, 1990), showed the complete absence of CD5 mRNA in some polyreactive clones. This was therefore consistent with our proposition that autoantibodies and multispecific antibodies are not restricted to the CD5 B cell subset.

These findings are in contrast to others in which autoreactive and polyreactive antibodies, derived from adult B lymphocytes, showed that this appeared to be an exclusive property of the CD5+ B cell subset (Casali et al, 1987 and Hardy et al, 1987).

Is there really a fundamental difference between adult and cord blood CD5+ and CD5- B cells ?

### 3. The nature of cord blood CD5+ and CD5- polyreactive antibodies

Clones of IgM secreting cells display various patterns of reactivity against the panel of antigens in this study.

Since all the clones secrete IgM, the variation in the antigen specific clones could reflect differences in the avidity of the antibody for a given antigen and as such, individual clones seem to display unique avidity patterns for the various antigens in the panel. Similar results have been obtained with multispecific monoclonal antibodies secreted by murine hybridomas (Ternyck et al, 1986).

These observations, plus the fact that both CD5+ and CD5- derived clones contained large numbers of IgM secreting clones that did not show any reactivity to the panel of antigens, lead one to argue against non-specific "stickyness" of monoclonal IgM molecules.

To confirm the specificity of the activity observed in the direct binding assays, inhibition studies were performed. In many of the clones assayed, binding of polyreactive IgM antibodies to IgG, ssDNA and collagen was blocked only by high concentrations of soluble ligand, suggesting the avidities of the IgM, from both subsets, are low.

Preliminary data would indicate that with cord blood IgM, soluble DNA blocks the binding of certain multispecific monoclonals to DNA and to heterologous ligand (IgG). Cross blocking has also been shown by others in some cases of polyspecificity (Dighiero et al, 1987, Logtenberg et al, 1987, Shoenfeld et al, 1983 and Ternyck et al, 1986, Casali and Notkins, 1989).

The molecular basis underlying the behaviour of polyreactive antibodies, relies on the notion that their antigen binding sites are considerably greater than that needed to accomodate only a single epitope. Furthermore, they are complementary to a number of different ligands (Richards et al, 1975 and Amit et al, 1986). Comparison of amino acid sequence data of murine light chains (Schlomchik et al, 1986), has introduced the idea that framework rather than complementarity determining regions may confer RF activity.

Therefore, unconventional binding sites may contribute to multispecificity and may explain why crossblocking can sometimes be demonstrated. It has been suggested that polar amino acids play an important role in the interaction of polyreactive natural antibodies (mainly arginine and lysine), with the various antigens (mainly glutamic and aspartic acids) ( reviewed by Avrameas, 1988). This is discussed in more detail in Chapter 7.

In conclusion, the results from these studies would indicate that auto and polyspecificity, is not restricted to the surface CD5 positive cord blood B cells.

Nevertheless, any putative CD5- detected B cell population, may be derived from the CD5+ population simply by loss of surface CD5 expression. It is yet to be established if activation/selection and subsequent loss of the CD5 surface molecule is an ongoing process in the fetal environment.

The definitive evidence of the origin of these clones, would of course, be the presence of CD5 molecules on the cell surface. Only B cells from one clone showed evidence of

surface CD5 by immunofluorescence (data not shown), so indicating that immortalization by EBV may "down-regulate" the surface expression of CD5.

# Chapter 5

## Expression of cross-reactive idiotopes (CRI) associated with $V_L$ and $V_H$ gene family products on IgM from CD5+ and CD5- cord blood B cells

## 5.1 INTRODUCTION

It was shown in Chapter 4, that cord blood B cells, rich in a CD5 expressing population, after immortalization with Epstein-Barr virus (EBV) produce IgM antibodies which exhibit auto and polyreactivity.

Furthermore, clones derived from both presorted CD5+ and CD5- cord blood cells exhibit auto and polyspecificity, with a higher frequency of anti-Fc and ssDNA activity in the CD5+ group.

A number of studies in mice have suggested that CD5+ B cells might differ in the expression of variable region genes from their conventional counterparts (Forster et al, 1988, Andrade et al, 1989, Jeong et al, 1990).

The definitive data for usage of variable region genes, must come from detailed sequence analysis. However, since idiotypic determinants are frequently associated with the binding site, idiotypic analysis can be a sensitive detector of differences among antibodies that have the same or similar specificities, such as rheumatoid factor (RF). Furthermore, a number of studies have suggested that CRI are the products of germ-line or minimally mutated germ-line genes (Kipps et al, 1988i).

In this study I have examined the clonal utilization of a) Light chain ($V\kappa$) and b) Heavy chain (VHI, VHIII, VHIV) subgroups, determined by their expression of certain CRI.

This was achieved using a panel of well characterised monoclonal antibodies, raised against RF paraproteins and defining idiotopes associated with these particular $V\kappa$ and VH gene family products (described in Chapter 1 and 2).

IgM from CD5+ and CD5- derived cord blood EBV B cell

clones was also examined for binding to Staphylococcal Protein A (SpA).

SpA is a bacterial membrane protein which binds human IgG1,2 and 4 at a site on the Fc part of the $C\gamma2$-$C\gamma3$ domain interface region (Langone et al, 1982 and Deisenhofer et al, 1981). Since it has recently been demonstrated that SpA also binds to monoclonal IgM molecules possessing, a VHIII heavy chain subgroup and that nearly all VHIII IgM paraproteins bind SpA (Sasso et al, 1989), this was used as a marker for the utilization of VHIII genes within the groups of cord blood clones.

## 5.2. RESULTS.

### 5.2.1. EXPRESSION OF CROSS-REACTIVE IDIOTOPES (CRI) ASSOCIATED WITH KAPPA LIGHT CHAIN GENE FAMILY PRODUCTS.

The IgM carrying B cell EBV clones, established, as described in Chapter 4, were examined for binding to a panel of well characterized, (as described in Chapter 1), monoclonal antibodies, C7, C6 and 17.109 and associated with particular Vκ gene family products.

The monoclonal antibodies were used at optimal concentrations (described in *Materials and Methods*) to stain the B cell clones and the fluorescent B cells examined using a FACScan flow cytometer. In some cases, the IgM, secreted and collected from the same clones of B cells, was also examined in parallel by ELISA, for the expression of these CRI. (These studies were kindly performed by Dr. R.A. Mageed).

#### 5.2.1.1. Expression of VκIII (C7) and VκIIIb sub-subgroup (17.109) associated CRI, on cord blood and fetal liver clones.

Initially, a pilot study was performed to determine the frequency of expression the Vκ associated CRI, using the monoclonal antibodies, C7 and 17.109, by early B cells (fetal liver and cord blood), a high percentage of which I had determined, (see Chapter 3) expressed CD5, prior to EBV immortalization.

Of 3 fetal liver clones and 5 cord blood clones expressing kappa light chain, 3 cord blood B cells were of the VκIIIb subgroup, as shown by reactivity with the C7

monoclonal antibody, 2 of which also expressed the 17.109 CRI, (Table 5.1).

**TABLE 5.1 Idiotope expression of fetal liver and cord blood clones.**

| Origin of Kappa clones | IDIOTOPE (Subgroup of light chain association) | |
|---|---|---|
| | C7 (VκIII) | 17.109 (VκIIIb) |
| FETAL LIVER (n = 3) | 0 (0%) | 0 (0%) |
| CORD BLOOD (n = 5) | 3 (60%) | 2 (40%) |

Analysis of light chain was performed by ELISA. Analysis of idiotopes was performed by immunofluorescence and analysis with a FACScan flow cytometer, and In some cases, also by ELISA.

C7, idiotope associated with VκIII subgroup of light chain ; 17.109, idiotope associated with VκIIIb.

### 5.2.1.2. Expression of VκIII, VκIIIb and VκIIIb sub-subgroup associated CRI by CD5+ and CD5- derived cord blood clones.

Having established that cord blood B cells display IgM which utilizes VκIII subgroup of light chains, I wished to determine the frequency of clones expressing this subgroup derived from CD5+ and CD5- clones that had been established in Chapter 4.

Using the panel of monoclonal antibodies (C7, C6 and 17.109), it was found that, within the kappa expressing CD5+ clones, 22/34 (65%) expressed the $V_{\kappa}III$ (C7) subgroup of light chain, a frequency similar to the proportion of CD5- clones, 11/20 (55%) expressing this CRI (Fig. 5.1).

Similarly, the frequency of kappa expressing CD5+ clones expressing the $V_{\kappa}IIIb$ sub-subgroup of light chain (C6), was 21/34 (62%) compared with 9/20 (45%) of the CD5- clones (Fig. 6.1).

However, the frequency of kappa light chain clones expressing the 17.109 CRI was significantly higher ($p<0.025$) in the CD5+ clones, 11/34 (32%) compared with the CD5- clones, 1/20 (5%) (Fig. 5.1).

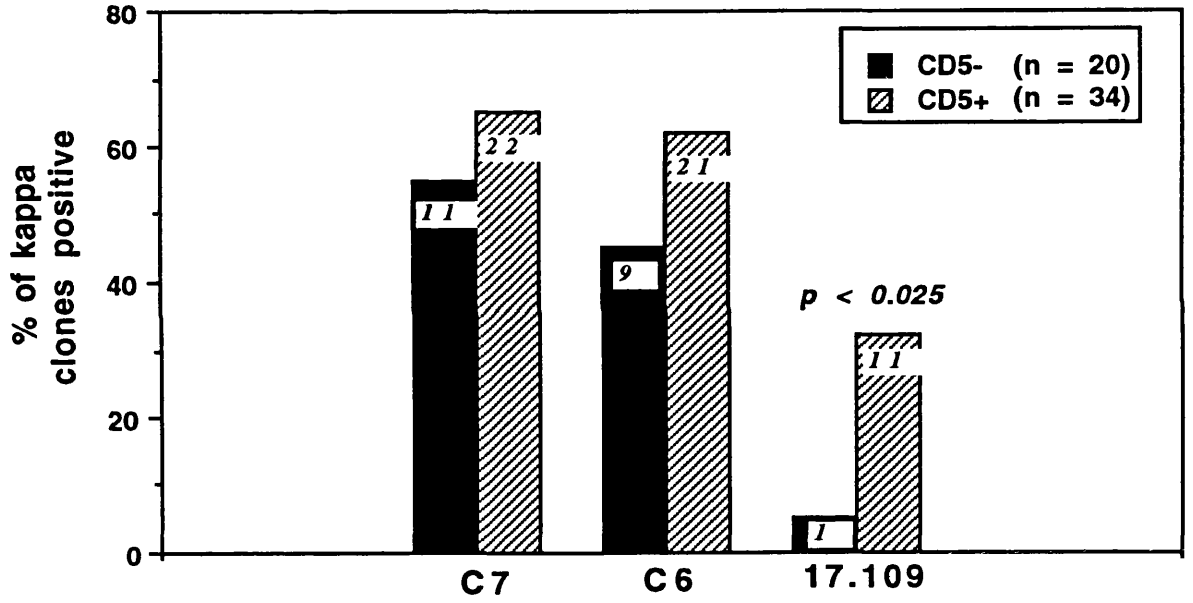


**FIGURE 5.1 EXPRESSION OF VκIII ASSOCIATED CROSS-REACTIVE IDIOTOPES (CRI) BY CD5+ AND CD5- CORD BLOOD CLONES.** Expression of CRI, (C7, C6 and 17.109) is expressed as the percentage of kappa light chain bearing clones, positive in 20 CD5- and 34 CD5+ clones and are represented as histograms. CRI were detected by flow cytometry (using a FACScan) and in some cases, also by ELISA. The p value is the result of the comparison of clones expressing the 17.109 CRI in the CD5+ and CD5- groups.

***Results are a cumulation of data obtained from 3 cord blood donors and are comparable to that data derived from any one of the donors, examined individually.***

## 5.2.2. EXPRESSION OF CROSS-REACTIVE IDIOTOPES ASSOCIATED WITH HEAVY CHAIN GENE FAMILY PRODUCTS.

The IgM carrying B cell EBV clones, established, as described in Chapter 4, were also examined for binding to a panel of well characterized, (as described in Chapter 1), monoclonal antibodies, associated with particular VHI, VHIII and VHIV gene family products.

The monoclonal antibodies were used at optimal concentrations (described in *Materials and Methods*) to stain the B cell clones and the fluorescent B cells examined using a FACScan flow cytometer. In some cases, the IgM, secreted and collected from the same clones of B cells, was also examined in parallel by ELISA, for the expression of these CRI. (These studies were kindly performed by Dr. R.A. Mageed).

### a) EXPRESSION OF VHI ASSOCIATED CROSS-REACTIVE IDIOTOPES (CRI).

#### 5.2.2.1. G6 and G8 expression by cord blood and fetal liver clones.

Preliminary experiments were performed on 9 fetal liver and 11 cord blood clones to establish the expression of the VHI associated CRI, G6 and G8.

IgM from 0/9 (0%) of the fetal liver clones examined expressed the conformational VHI associated idiotype, G8. G8, was however, expressed on IgM from 2/11 (18 %) of the cord blood clones, both of which carried lambda light chains (Fig.5.2).

The VHI associated idiotype, G6, was not found on any

of the clones included in this study (Fig.5.2).

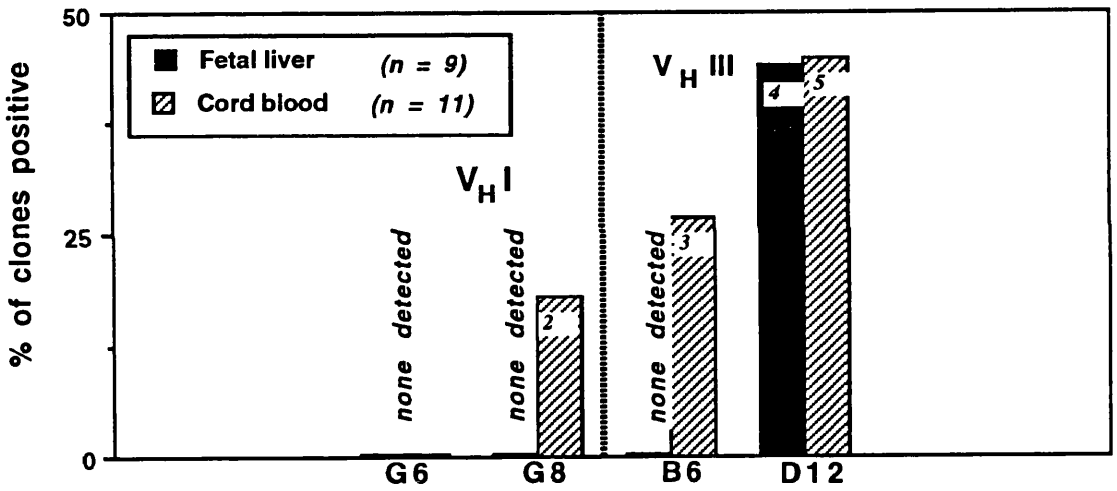


**FIGURE 5.2. EXPRESSION OF VHI AND VHIII ASSOCIATED CROSS-REACTIVE IDIOTOPES (CRI) BY CORD BLOOD AND FETAL LIVER B CELL CLONES.** Expression of G6 and G8 (VHI associated) and B6 and D12 (VHIII associated) CRI, are expressed as a percentage positive of 9 fetal liver and 11 cord blood clones and are represented as histograms. CRI were detected by immunofluorescence and flow cytometry (using a FACScan) and in some cases, also by ELISA.

***Results are a cumulation of data obtained from 3 cord blood donors and are comparable to that data derived from any one of the donors, examined individually.***

### 5.2.2.2. G6 and G8 expression by CD5+ and CD5- derived cord blood clones.

With the aim of investigating any relationship between the expression of these CRI and CD5 positivity, I examined the CD5+ and CD5- cord blood EBV B cell clones.

IgM from none of the 40 CD5+ or 45 CD5- clones examined expressed the VHI associated idiotype, G6 (Table 5.2).

However, 2 CD5+ kappa light chain clones expressed the G8 CRI, as did 2 CD5- kappa light chain clones, interestingly all belonging to the VκIII subgroup, as detected by monoclonal antibody, C7 (see Fig.6.6).

One lambda light chain clone expressing G8 was detected in the CD5- group (Table 5.2).

**TABLE 5.2 Expression of G6 and G8 by CD5+ and CD5- cord blood B cell clones.**

| CLONE ORIGIN | TOTAL NUMBER | NUMBER (%) CLONES EXPRESSING G6 | G8 |
|---|---|---|---|
| **CD5+** | | | |
| IgM κ | 29 | 0 (0%) | 2 (7%) |
| IgM λ | 11 | 0 (0%) | 0 (0%) |
| *Total* | 40 | 0 (0%) | 2 (5%) |
| **CD5-** | | | |
| IgM κ | 20 | 0 (0%) | 2 (10%) |
| IgM λ | 25 | 0 (0%) | 1 (4%) |
| *Total* | 45 | 0 (0%) | 3 (7%) |

Light chain restriction was performed by ELISA and analysis of idiotopes by both immunofluorescence and flow cytometry (using a FACScan) and by ELISA.
G6, G8, idiotopes associated with VHI.

## b) EXPRESSION Of VHIII ASSOCIATED CROSS-REACTIVE IDIOTOPES (CRI).

### 5.2.2.3. B6 and D12 expression by cord blood and fetal liver clones.

Initially, preliminary experiments were designed to investigate the expression of the VHIII associated CRI, B6 and D12 on the 9 fetal liver and 11 cord blood clones we had available.

4/9 (44%) of the fetal liver clones expressed the VHIII associated D12 idiotope and it was expressed by 5/11 (45%) of the cord blood clones analysed, 3 of these, in fact, co-expressed B6 (Fig. 5.2).

### 5.2.2.4. B6 and D12 expression by CD5+ and CD5- derived cord blood clones.

I next examined IgM collected from 40 of the CD5+ and 45 of the CD5- cord blood EBV clones I had produced (Chapter 4).

The VHIII associated idiotopes, B6 and D12 were present on IgM at the same frequency in both CD5+ and CD5- populations (Fig.5.3a).

An analysis of the light chain expression of these clones, shows that 5/40 (12.5%) of the CD5+ clones, coexpressed B6 and D12. Interestingly, although not significantly, this was restricted to kappa light chain, since no lambda clones examined expressed either of these VHIII associated idiotopes in the CD5+ group (Table 5.3).

Of IgM from 45 CD5- clones examined, 2, kappa clones co-expressed B6 and D12 and 3 lambda clones expressed D12

only (Table 5.3a).

### 5.2.2.5. VHIII gene family usage in CD5+ and CD5- clones, as determined by binding to Staphylococcal Protein A (SpA).

**a) Equal representation of VHIII gene family usage by CD5+ and CD5- cord blood clones**

IgM from 47 CD5+ and 45 CD5- cord blood clones was examined using ELISA and reverse passive haemagglutination assays, kindly performed by Dr. RA Mageed (and described in *Materials and Methods*).

Results showed that IgM from both CD5+, (16/47, 34%) and CD5-, (22/46, 48%) clones bound Staphylococcal Protein A (SpA) at a similar frequency, indicating similar usage of VHIII family genes (Fig.5.3b).

**b) Higher numbers of kappa clones, than lambda clones, derived from CD5- B cells, produce IgM which reacts with SpA.**

There was, overall, a trend, though not significant, towards higher numbers of kappa clones that bound SpA (24/51) than lambda clones (14/42), see Table 5.3b.

However, there was a significantly higher frequency of kappa clones in the CD5- group that bound SpA (12/18) when compared with the kappa clones, in the CD5+ group (12/33) ($p<0.05$).

Furthermore, significantly higher numbers of kappa clones than lambda clones bound to SpA, within the CD5- group (12/18 $\kappa$ compared with 10/28 $\lambda$, $p<0.02$), (Table 5.3b).

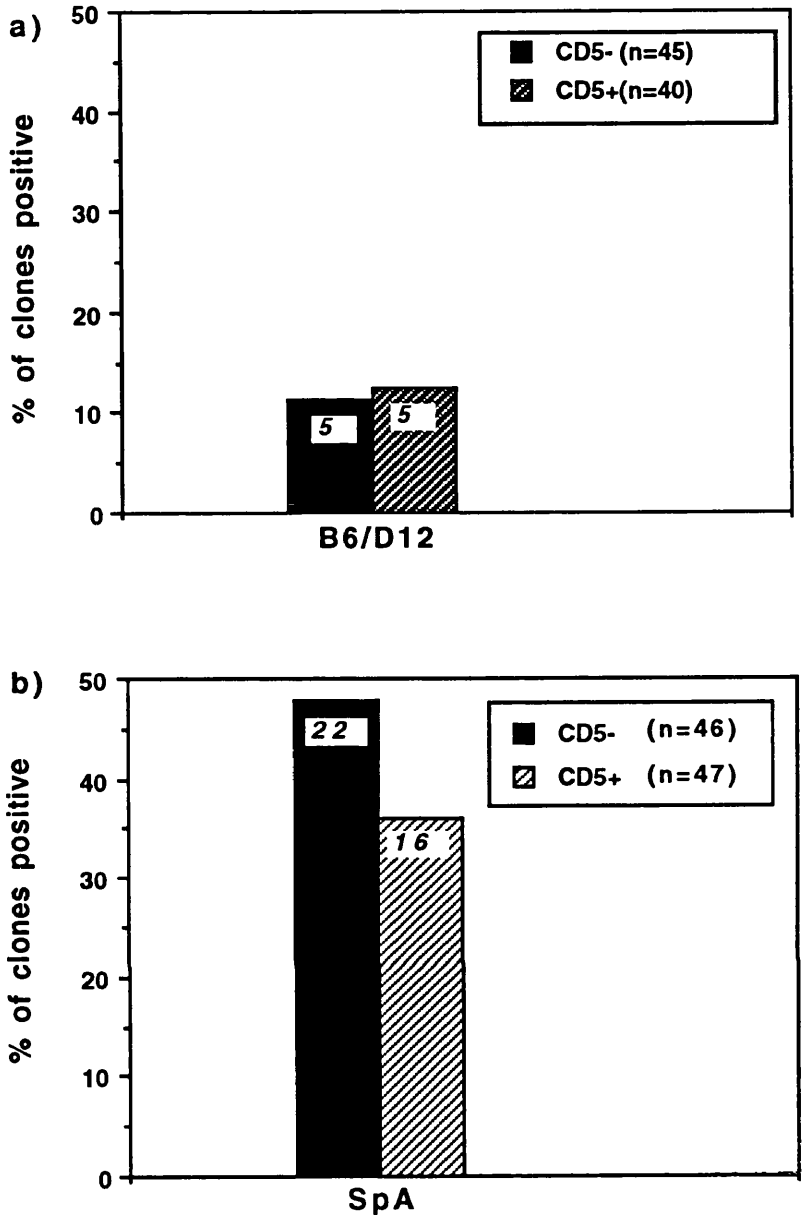


**FIGURE 5.3. FREQUENCY OF EXPRESSION OF VHIII ASSOCIATED CROSS-REACTIVE IDIOTOPES (CRI) (a) and BINDING TO STAPHYLOCOCCAL PROTEIN A (SPA) (b), BY CD5+ AND CD5- CORD BLOOD CLONES.** Expression of B6 and D12 (Fig.5.3a) and binding to SpA (Fig.5.3b) are expressed as the percentage positive of 40 CD5+ and 45 CD5- and 47 CD5+ and 46 CD5- cord blood B cell clones, respectively, and are represented as histograms. CRI were detected by both ELISA and flow cytometry (using a FACScan). Binding to SpA was detected by ELISA and haemagglutination assays (see *Materials and Methods*).

***Results are a cumulation of data obtained from 3 cord blood donors and are comparable to that data derived from any one of the donors, examined individually.***

**TABLE 5.3. Expression of VHIII associated cross-reactive idiotopes (CRI) (a) and binding to Staphylococcal Protein A (SpA) (b), of IgM from CD5+ and CD5- cord blood B cell clones.**

| CLONE ORIGIN | TOTAL NUMBER | NUMBER (%) CLONES EXPRESSING : B6 | D12 | TOTAL NUMBER | NUMBER (%) CLONES BINDING TO: SpA |
|---|---|---|---|---|---|
| CD5+ | a) | | | b) | |
| IgM κ | 29 | 5 (17%) | 5 (17%) | 33 | 12 (36%) * |
| IgM λ | 11 | 0 (0%) | 0 (0%) | 14 | 4 (28.5%) |
| *Total* | *40* | 5 (12.5%) | 5 (12.5%) | *47* | 16 (34%) |
| CD5- | | | | | |
| IgM κ | 20 | 2 (10%) | 2 (10%) | ** 18 | 12 (67%) * |
| IgM λ | 25 | 0 (0%) | 3 (12 %) | ** 28 | 10 (36%) |
| *Total* | *45* | 2 ( 4%) | 5 (11%) | *46* | 22 (48%) |

Analysis of light chains was performed by ELISA and idiotopes by both ELISA and flow cytometry (using a FACScan).Binding to Staphylococcal Protein A (SpA) was performed by ELISA and haemagglutination assays by Dr RA Mageed (see *Materials and Methods*). B6,D12 ID, idiotopes associated with VHIII.

* $p<0.05$. The p value is the result of the comparison of the percentage of kappa clones binding SpA in the CD5+ and CD5- groups.

** $p<0.02$. The p value is the result of the comparison of the percentage of kappa and lambda clones binding Spa in the CD5- group.

## c) EXPRESSION OF VHIV ASSOCIATED CROSS REACTIVE IDIOTOPES (CRI).

### 5.2.2.6. A high frequency of VHIV gene family expression by CD5+ and CD5- derived cord blood cells.

Using the monoclonal antibodies, Lc1, 9G4 and R2.1A2, described in Chapter 1 and that are specific for VHIV associated CRI, I examined 46 CD5+ and 47 CD5- of our established (see Chapter4) cord blood EBV B cell clones.

Overall, IgM form a relatively higher proportion, though not significantly so, of CD5+ clones (18/46, 39%) express VHIV genes compared with their CD5- counterparts (12/47, 26%), Table 5.4.

Interestingly, the frequency of expression of the VHIV CRI by kappa light chain clones belonging to the CD5+ population (14/30, 47%), was much higher than the frequency of expression by the lambda clones (4/16, 25%), in the same group of B cells (Table 5.4). This higher representation was not observed within the CD5- kappa and lambda light chain bearing cells.

### 5.2.2.7. High frequency of expression of Lc1 by IgM from CD5- B cells.

It appeared that IgM from the CD5- group of B cell clones expressed a slightly higher frequency of the Lc1 CRI (12/47, 26%) when compared with that expressed by the CD5+ population (7/46, 15%). (Table 5.4).

Furthermore, there was a trend towards significance ($p<0.1$), that higher numbers of lambda clones in the CD5- group, expressed the Lc1 CRI (1/16), when compared with the

number of lambda clones that expressed Lc1 in the CD5+ group (8/28). In fact, if one only considers the Lc1 positive clones, then a comparison can be drawn between the lambda clones in the CD5- group (8/12) and lambda clones in the CD5+ group (1/7) and the difference is significant ($p<0.02$).

### 5.2.2.8. Co-expression of CRI recognised by R2.1A2 and 9G4.

In all cases examined, it appeared that both R2.1A2 and 9G4 reacted with the same IgM molecules and were associated with kappa light chain expression in 8/11 (73%) of the clones (Table 5.4), (although not belonging to the VκIII subgroup (see 5.2.2.9 below)).

### 5.2.2.9. High frequency of association of the CRI recognised by Lc1 antibodies and VκIII subgroup of light chain, in the CD5+ clones.

Interestingly, although 65% of the CD5+ kappa clones, expressed the VκIII subgroup, identified by monoclonal C7, only 1/8 (12%) of the R2.1A2/9G4 positive clones, were found in association with this subgroup of light chain (see Fig.5.6)

In comparison, 6/6 (100%) of the Lc1 positive clones, were found to be associated with the VκIII subgroup, in this population (see Fig.5.6).

### 5.2.2.10. Selective expression of a subset of VHIV genes characterised by the expression of R2.1A2 and 9G4 CRI.

The results indicated on Table 5.4 and Fig.5.4 show that, whereas IgM from 11/46 (24%) of the cord blood clones

derived from the CD5+ population coexpressed both R2.1A2 and 9G4, no (0%) CD5- clones expressed either of these CRI ($p<0.01$).

**TABLE 5.4 Frequency of expression of VHIV associated cross-reactive idiotopes (CRI) by IgM from CD5+ and CD5- cord blood clones.**

| CLONE ORIGIN | TOTAL NUMBER | NUMBER (%) CLONES EXPRESSING Lc1 | R2.1A2 | 9G4 |
|---|---|---|---|---|
| **CD5+** | | | | |
| IgM κ | 30 | 6 (20%) | 8 (27%) | 8(27%) |
| IgM λ | 16 | *1 (6%) | 3 (19%) | 3 (19%) |
| *Total* | *46* | 7 (15%) | 11 (24%) | 11 (24%) |
| **CD5-** | | | | |
| IgM κ | 19 | 4 (21%) | 0 (0%) | 0 (0%) |
| IgM λ | 28 | *8 (29%) | 0 (0%) | 0 (0%) |
| *Total* | *47* | 12 (26%) | 0 (0%) | 0 (0%) |

Analysis of light chains was performed by ELISA and idiotopes by both ELISA and flow cytometry (using a FACScan).

Lc1, R2.1A2 and 9G4, idiotopes associated with VHIV.

* $p<0.02$. The p value is the result of the comparison between the Lc1 positive clones that also express lambda in the CD5+ and CD5- groups.

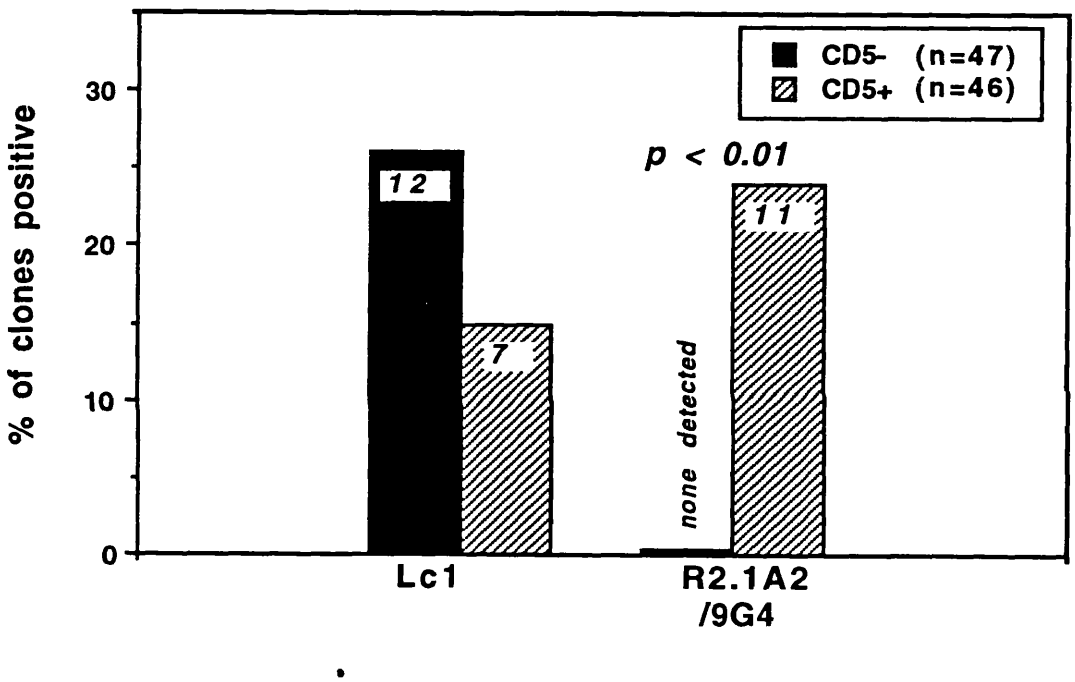


**FIGURE 5.4. FREQUENCY OF EXPRESSION OF VHIV ASSOCIATED CROSS-REACTIVE IDIOTOPES (CRI) BY CD5+ AND CD5- CORD BLOOD B CELL CLONES.** The expression of Lc1, R2.1A2 and 9G4 on IgM from 47 CD5- and 46 CD5+ cord blood B cell clone are expressed as percentage positive and are represented as histograms. CRI were detected by immunofluorescence and flow cytometry (using a FACScan) and by ELISA. The p value is the result of the comparison between the percentages of clones positive for R2.1A2/9G4 in the CD5+ and CD5- groups.

***Results are a cumulation of data obtained from 3 cord blood donors and are comparable to that data derived from any one of the donors, examined individually.***

### 5.2.2.11. Expression of VHI, VHIII and VHIV gene families in the CD5+ and CD5- cord blood B cell clones.

Fig. 5.5 is a summary of the representation of VHI,III and IV gene families, as determined by the expression of associated CRI and the binding to Staphylococcal Protein A. It was determined from analysis of the 43 CD5- (Fig5.5a) and 40 CD5+ (Fig.5.5b), cord blood clones.

Eighty-four and 87.5% of the CD5- and CD5+ clones respectively, are accounted for in this study.

Although there was a trend towards a higher representation of VHIV associated CRI in the CD5+ group (45%), compared with the CD5- group (28%), this was not statistically significant and no other significant differences, in the representation of VHI or VHIII associated CRI could be determined between the two subpopulations of cells.

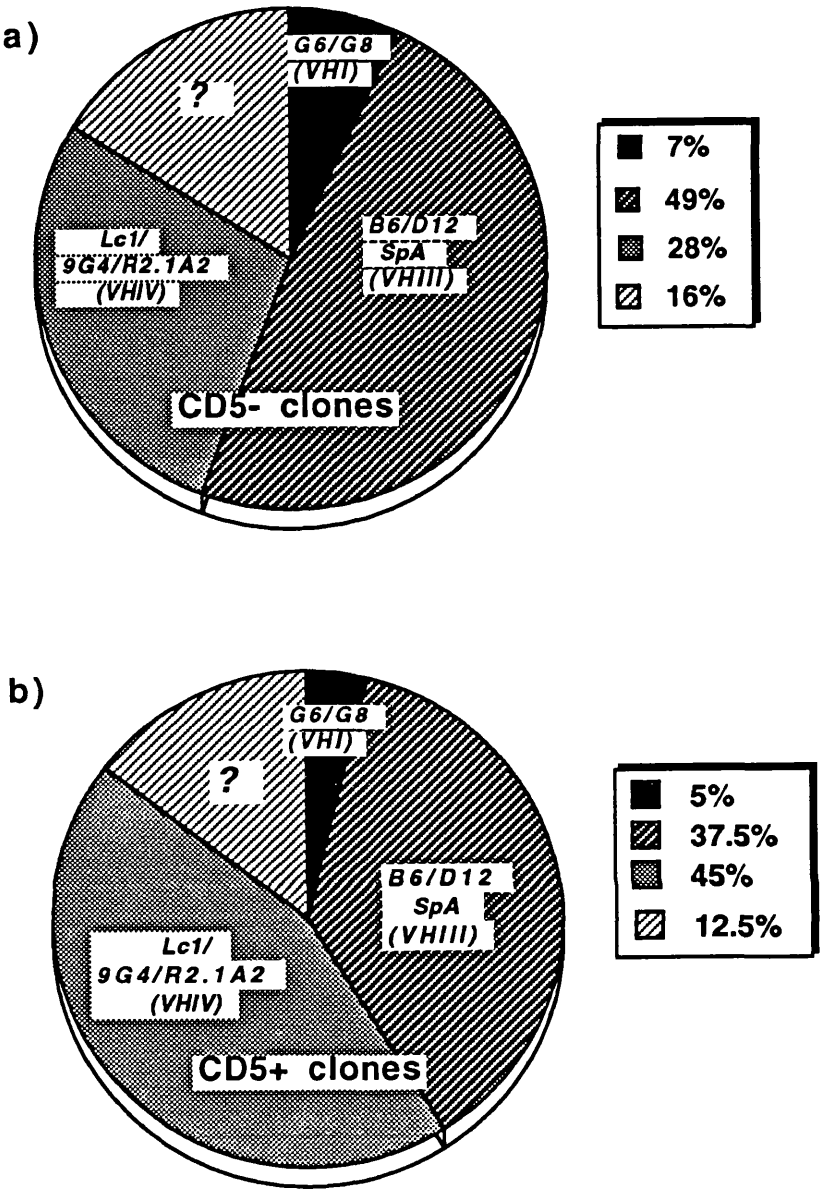


**FIGURE 5.5 SUMMARY OF EXPRESSION OF VH GENE FAMILIES BY CD5+ AND CD5- B CELL CLONES IN THE STUDY.** The number of clones expressing G6/G8 (VHI), B6/D12 (VHIII) and binding to Staphylococcal Protein A (SpA) (VHIII) and Lc1/9G4/R2.1A2 (VHIV), are represented as percentages positive of 43 CD5- (Fig.6.5a) and 40 CD5+ (Fig.6.5b) cord blood B cell clones, and are represented as Venn diagrams for each group.

***Results are a cumulation of data obtained from 3 cord blood donors and are comparable to that data derived from any one of the donors, examined individually.***

## 5.2.3. THE SPECIFICITIES OF CD5+ AND CD5- CORD BLOOD B CELLS IN RELATION TO THEIR LIGHT AND HEAVY CHAIN FAMILIES.

The data presented in this section is a compilation of the results obtained in this study.

Fig.5.6 and Table 5.5, illustrates the association of kappa and lambda light chains with particular heavy chain families and their relationship with autoreactivity.

### 5.2.3.1. Specificity of 17.109 positive cord blood B cell clones

Of 11 CD5+ clones bearing the 17.109 CRI, 8 were tested for co-expression with VH associated CRI's.

The results showed that 3/8 expressed VHIII gene family products and 5/8, VHIV associated CRI's (Fig.5.6a).

No differences in specificity could be determined, as 7/8 of the clones had specificity for the Fc of IgG. Six of these were also polyreactive binding to a variety of antigens including ssDNA, Collagen I/II, mitochondria and cardiolipin (data not shown).

The 1 CD5- clone which expressed 17.109 was not autoreactive and, through binding to Staphylococcal Protein A (SpA), was found to be expressed in association with VHIII gene products.

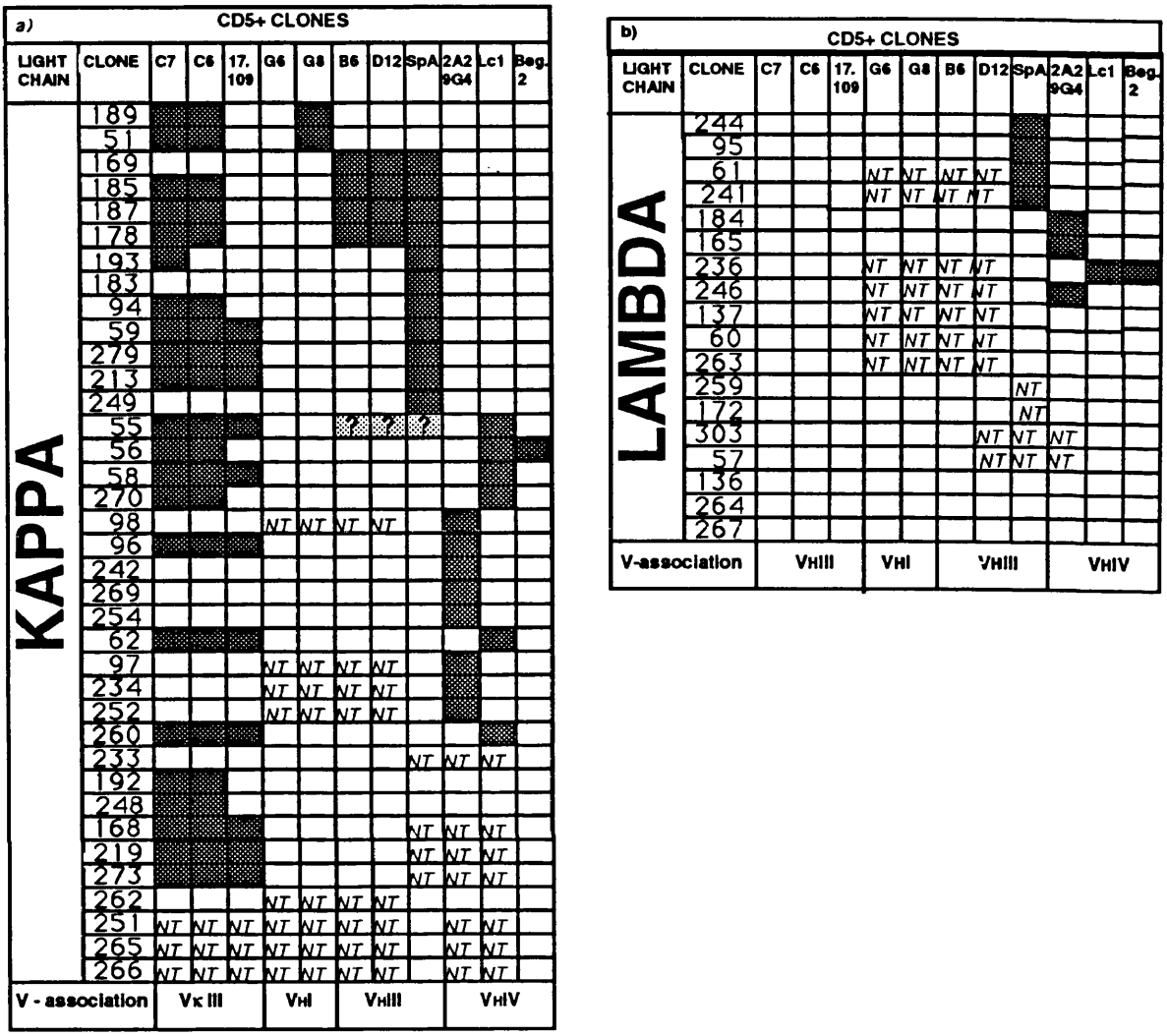


**FIGURE 5.6a AND b. EXPRESSION OF IDIOTOPES AND LIGHT CHAIN SUBGROUPS IN CD5+ CORD BLOOD CLONES IN THE STUDY.** IgM from CD5+ kappa (a) and lambda (b) cord blood clones were examined for the expression of idiotope and light chain subgroups by ELISA and by flow cytometry, as described in *Materials and Methods.* Both assays were performed in parallel and the results identical.
C7,C6 and 17.109 (VκIII associated cross-reactive idiotopes (CRI), G6 and G8 (VHI associated CRI), B6 and D12 (VHIII associated CRI), SpA (Staphylococcal Protein A), Lc1,R2.1A2/9G4 (2A2/9G4) and Beg2 (Beg2 idβ) (VHIV associated CRI).

positive, not known, NT; not tested

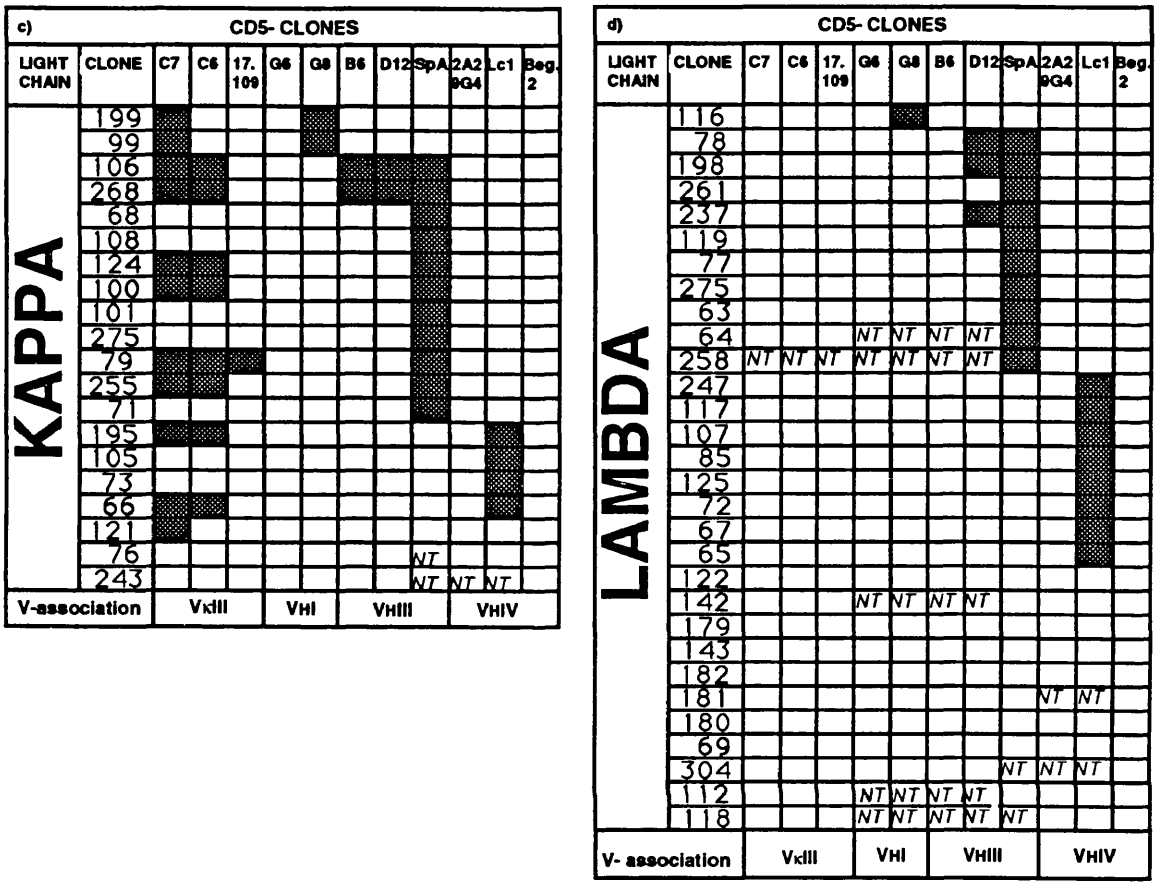


**FIGURE 5.6c AND d. EXPRESSION OF IDIOTOPES AND LIGHT CHAIN SUBGROUPS IN CD5- CORD BLOOD CLONES IN THE STUDY.** IgM from CD5- kappa (c) and lambda (d) cord blood clones were examined for the expression of idiotope and light chain subgroups by ELISA and by flow cytometry, as described in *Materials and Methods.* Both assays were performed in parallel and the results identical.

C7,C6 and 17.109 (VκIII associated cross-reactive idiotopes (CRI), G6 and G8 (VHI associated CRI), B6 and D12 (VHIII associated CRI), SpA (Staphylococcal Protein A), Lc1,R2.1A2/9G4 (2A2/9G4) and Beg2 (Beg2 idβ) (VHIV associated CRI).

positive, not known, NT; not tested

**5.2.3.2. CD5+ and CD5- B cells utilize the same or similar VH family gene products and both can both be autoreactive.**

**a)** Fig.5.6 and Table 5.5 shows that although both CD5+ and CD5- clones, expressed the VHI associated CRI, recognised by monoclonal antibody, G8, in association with VκIII (C7), this did not appear to determine auto/polyreactivity.

Furthermore, the CD5- lambda clone that was G8 positive, (#116), displayed extensive auto/polyreactivity that included specificities for the Fc of IgG, ssDNA, collagen, vimentin, cytokeratin and perinuclear antigen (see Fig.4.11b).

**b)** The specificities of the CD5+ and CD5- clones which expressed VHIII associated CRI, are also shown on Table 5.5.

The data indicate that no significant differences were detectable between the two subgroups of B cells concerning their auto and polyreactivity, when they expressed VHIII family genes.

**c)** In this study, the expression of heavy chains from the VHIV family of genes was associated with antibodies that were auto and polyreactive in both the CD5+ and CD5- populations.

Interestingly, a high percentage of the CD5+ cord blood clones that were R2.1A2/9G4 positive, were auto and polyreactive, (91% ; 10/11), (Table 5.5). This specificity could not be related to expression of the VκIII subgroup of light chain (Fig 5.6a), since only 1 clone was found to be R2.1A2/9G4 and C7 positive.

**TABLE 5.5 Frequency of autoreactivity of CD5+ and CD5- B cells, expressing specific VH family-associated cross-reactive idiotopes (CRI).**

| | CD5+ | | | | | CD5- | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | VHI | VHIII | | VHIV | | VHI | VHIII | | VHIV | |
| *SPECIFICITY* | G8 | SpA | B6/ D12 | Lc1 | R2.1A2 | G8 | SpA | B6/ D12 | Lc1 | R2.1A2 |
| Fc | 1/2 | 8/15 | 3/4 | 3/7 | 10/11 | 2/3 | 6/21 | 3/5 | 5/12 | 0 |
| ssDNA | 1/2 | 6/15 | 3/4 | 5/7 | 10/11 | 2/3 | 4/21 | 1/5 | 5/12 | 0 |
| polyreactive ≥ 2 antigens | 1/2 | 6/15 | 3/4 | 3/7 | 10/11 | 3/3 | 8/21 | 4/5 | 6/12 | 0 |

Fc, Fc of IgG, ssDNA, single-stranded DNA, polyreactive, >2, those clones found to display more than two different (auto)specificities from the panel tested.
G8 (VHI associated CRI), SpA (Staphylococcal Protein A), B6/D12 (VHIII associated CRI), Lc1, 9G4/R2.1A2 (VHIV associated CRI)

## 5.3 DISCUSSION

My earlier studies, have shown that a high frequency of EBV immortalized cord blood B cell clones produce IgM antibodies which display extensive autoreactivity for IgG Fc (rheumatoid factor, RF), and mainly are all polyreactive.

In this study, IgM produced by the clones was analysed for the expression of cross-reactive idiotopes (CRI) associated with RF paraproteins and characteristic of defined VH and Vκ subgroups.

In general, a large number of CD5+ and CD5- cord blood clones are likely encoded by germline genes. Furthermore, in particular, I observed, the exclusive expression of specific VHIV family genes and a selective expression of the Vκ325 germ-line gene, by IgM from the CD5+ B cell subset.

Analysis of the Vκ and VH gene family associations of the CD5+ and CD5- clones and their relative specificities are discussed below.

### 1. Expression of VL subgroups

#### *a) VκIII associated products*

In comparison with the level detected in normal IgM, (Crowley et al, 1988 and Mageed et al, 1988), a much higher percentage of unseparated neonatal B cell clones, in this study, expressed the VκIIIb associated idiotope (id), 17.109 .

An extension of these studies, examined cord blood clones, derived from CD5+ and CD5- cells. I had previously shown that, the frequency of kappa light chain expressing clones, was highest in those clones derived from CD5 B cells. In these studies, I observed that, whereas, IgM from kappa

bearing cells from both populations, expressed high levels of the VκIII subgroup, significantly more clones from the CD5+ subset, reacted with the 17.109 monoclonal antibody, than CD5- clones. Other studies have demonstrated a relatively high frequency of VκIII subgroup expression in B cells from patients with chronic lymphocytic leukemia (CLL), which express surface CD5 (Kipps et al, 1987ii and 1988ii).

Since the 17.109 antibody recognises a product of a germ-line gene, Vκ325, which is associated with the VκIIIb sub-subgroup of light chains (Radoux et al, 1986), these observations might suggest a selective expansion of CD5 B cells during development. It has recently been shown that, the products of the Vκ325 gene, are expressed by many normal cells early in ontogeny in the human spleen (Kipps et al, 1990). In fact, normal CD5+ B cells in the mantle zone and interfollicular region, surrounding the germinal centres of human tonsil and spleen, were shown to contain more cells bearing the 17.109 CRI than CD5- B cells (Kipps et al, 1989).

The conservation of the primary sequence of variable region genes encoding the 17.109 CRI, suggest the proteins they encode are important and so is surely related to the autoreactivity of the antibody these variable region genes encode. The 17.109 id is highly associated with RF activity (Crowley et al, 1988 and Silverman et al, 1988) and consistent with this, I found that the majority of clones, expressing 17.109 id produced IgM with specificity for Fc of IgG.

However, large numbers of both CD5+ and CD5- clones expressing other kappa and even, lambda light chains have specificity for RF. This would infer that the gene coding for the 17.109 CRI is only one of several contributing to the

binding of IgG.

## 2. Expression of VH associated idiotopes

### *a) VHI associated CRI*

The VHI associated and conformation dependent CRI, recognised by the antibody, G8 was found on both CD5+ and CD5- B cell clones at the same frequency.

Interestingly, however, the idiotope, recognised by the G6 antibody, was not found on any of the IgM examined in these studies. The idiotope, identified with the G6 monoclonal antibody, is known to be encoded for by the 51P1 germline gene (Kipps et al, 1989). Immunohistological studies using tissue sections of fetal spleen, identified small clusters of G6 positive B cells in the primary follicles (Kipps et al, 1990). It is not unreasonable to propose that G6 positive B cells are not transformable by EBV. On the other hand, our data (Lydyard et al, 1990) and that of others (Kipps et al, 1990), have identified CLL B cell clones expressing a relatively high frequency of G6 positivity, thus indicating that at least clones immortalized by an oncogenic event can express this CRI. However, since all the clones in the study were derived from only three cord blood samples, this may indicate a polymorphism in the expression of this CRI within the normal cord blood population. In fact, B lymphocytes from 10% of adults fail to express G6 (Kipps, personal communication).

17.109 positivity has been shown to be often associated with VHI in around 20% of B CLL patients (Kipps et al, 1989). Other studies have demonstrated co-expression of the G6 and 17.109 CRI by many normal B cells early in ontogeny in the human spleen (Kipps et al, 1990). It would appear that,

certain combinations of appropriately rearranged immunoglobulin light and heavy chains may generate immunoglobulin with antiself reactivity. In this study, despite the high degree of specificities directed towards self components, none of the 17.109 clones were associated with either G6 or G8 idiotopes (VHI).

*b) VHIII associated CRI and binding to SpA*

The high frequency of VHIII family expression, by early B cells, examined in these studies, is in accordance with the frequency of the VHIII family, expressed in adult IgM (Forre et al, 1989).

Furthermore, the VHIII associated CRI, recognised by the monoclonal antibodies, B6 and D12 were equally represented among the CD5+ and CD5- clones examined and in most cases, coexpressed. However, it is interesting, that in a few instances B6 and D12 were not co-expressed, particularly by the lambda bearing clones. Similarily, co-expression of B6 and D12, was not observed, in the fetal liver clones I examined. It is likely that the gene coding for the idiotope, recognised by the D12 monoclonal antibody, is expressed earlier in B cell development, than the gene coding for both. Indeed a restriction, early in ontogeny, in the usage of the genes associated with these proteins, may result in an important physiological function.

However, these idiotopes per se are not sufficient to confer RF activity on IgM antibodies of the VHIII family. Analysis of the specificities of the VHIII expressing clones revealed that auto and polyreactivity could be achieved using kappa or lambda chains and more significantly, many B6/D12

clones displayed no such autoreactivity, at least with the panel of autoantigens used.

Although germline encoded, CRI may be the product of more than one VH gene, therefore B6 and D12 may be markers for a number of VHIII germline gene encoded products, defining sub-subgroups of the VHIII gene family.

Clearly there was an association between SpA and VHIII associated CRI (B6 and D12), and not VHI or VHIV associated CRI expression. This is consistent with reports concerning the binding of SpA with VHIII proteins (Sasso et al, 1989).

Immunoglobulin from relatively high numbers of B cell clones from both the CD5+ and CD5- groups bound SpA, particularly those expressing kappa light chains. Furthermore, the predominance of kappa clones in the CD5- population, that bound SpA, might be interpreted as an indication of a specific cellular selection process, that has taken place on the basis of their antigenic receptors during development. The CD5- phenotype may have arisen as a consequence of this. That these cells may be directed towards a particular specificity could not be determined as, both CD5+ and CD5- clones utilising VHIII family associated genes, as determined by SpA binding, showed auto and polyreactivity to the same degree.

The trend towards a decreased binding of SpA by IgM produced by the CD5+ derived clones, most probably reflects the relative use of other VH families, by this subpopulation. However, it has been demonstrated that SpA shows highly selective binding to IgG, for example IgG3 binds weakly (reviewed by Boyle, 1990) and it would be interesting to analyse the isotypes of the antibodies in my studies.

Furthermore, the affinity of binding SpA by some of these monoclonals may be a question to consider.

The VHIII family is the largest of the human VH families, containing at least 100 genes and probably comprises many subfamilies (Souroujon et al, 1989). Therefore our result indicating a high percentage of both CD5+ and CD5- cells expressing VHIII family genes is consistent with the high percentage of polyclonal IgM using VHIII in the serum of adults (Crowley et al, 1990). Recent in situ hybridisation experiments demonstrated that 52% of peripheral blood lymphocytes express VHIII genes (Guigou et al, 1990).

Reactivity with the monoclonal antibody B6 is fairly common amongst SpA positive clones in this study (about 25%) and has been shown to inhibit the binding of monoclonal RF to IgG. Furthermore, SpA has been shown to inhibit RF binding to the monoclonal B6 (Crowley at al, 1990). This ability of SpA to block an idiotype-anti-idiotype interaction, either by steric hinderance or specific inhibition, has potential importance in the interaction of microbial proteins (Fleischer and Schrezenmeier et al, 1988).

Mammalian VH gene sequences are highly conserved and homology is greater between genes of equivalent VH families from different species, than between genes from different VH families of the same species (Rechari et al, 1983). The human VHIII corresponds to the murine VH7183, VHJ606, VHS107 and VHX24 families (Lee et al, 1987). It is possible that the nucleotides which encode the SpA binding site on human VHIII IgM are also conserved on these murine families and in fact, SpA binding has been reported in murine monoclonal IgM molecule (MacKenzie et al, 1978 ).

It seems possible that the genes belonging to the VHIII family have been selected for and conserved as a primitive defence against microrganisms including Staphylococcus.

*c) VHIV associated CRI*

Using the monoclonal antibodies, Lc1, R2.1A2 and 9G4, my results revealed, that the representation of VHIV genes in cord blood B cell clones, is considerably higher than would be predicted for this relatively small family of genes.

However the frequency of VHIV gene usage in fetal liver has been estimated at 21% (Schroeder et al, 1987), a frequency not disimilar to that observed in this study. In addition, a high frequency of VHIV usage in EBV immortalized adult CD5 B cell clones has been reported (Sanz et al, 1989).

Furthermore, a higher proportion of clones, derived from the CD5+ cord blood B cells, express VHIV genes compared to their CD5- counterparts.

Remarkably however, whereas CD5+ B cells express two identical CRI, R2.1A2 and 9G4, expressed on the same IgM molecules, CD5- B cells did not, ever, express either of them. This would suggest that a significant proportion of CD5+ B cell clones produce IgM derived from a single gene or a small number of genes within the VHIV family.

Conversely, higher numbers of CD5- clones, particularly lambda bearing, expressed Lc1, compared with the CD5+ group. This may reflect again, a selective pressure on the programmed expression of these VH genes during development (Perlmutter et al, 1985).

The VHIV family probably contains a small number (≤10) of genes with a high degree of internal homology. It is

likely then that the germline repertoire would encode a relatively few CRI. Although the three CRI used in this study cannot be formally assigned to a particular VH family, data has indicated an association of these CRI with different germline genes (Silverman et al, 1986 and Sanz et al, 1989). Lc1 may recognise a CRI and be a phenotypic marker of 71.2 and 71.4 and other related genes, whilst R2.1A2/9G4 may be markers for the 4-21 and related genes (Thompson et al, 1991 and Pascual et al, 1991). These two sets of germline genes might represent two different sub-families within the VHIV family (Mageed et al, 1991).

Despite the high frequency of VκIII bearing clones in the CD5+ group examined, in no clone could we demonstrate 17.109 or VκIII expression in association with either R2.1A2 or 9G4. An association of heavy chains from the VHIV family with VκIII subgroup of light chains has previously been reported in cold agglutinins (Silverman et al, 1988).

Finally, with regard to specificity, virtually all of the CD5+ clones that exclusively expressed the R2.1A2/9G4 epitopes, were polyreactive, suggesting a high degree of homology within this group. That these are really individual, different clones is being established and preliminary data of nucleotide sequencing (see Chapter 8), suggests that they are.

Interestingly, the R2.1A2 monoclonal antibody is a phenotypic marker for a CRI associated with anti-I carbohydrate antigen specificity. The reactivity of the clones in the study, with respect to this antigen is being investigated and preliminary data has shown that 5 R2.1A2/9G4 positive clones, but not 4 Lc1 positive clones, reacted with the I-antigen on red blood cells (Stevenson et al, unpublished). In

view of recent reports that an aspartic acid residue at position 31, is thought to be critical to the recognition of the red cell antigen (Pascual et al, 1991), sequence analysis of these clones may reveal the genetic basis governing such CRI expression.

# Chapter 6

## Expression of Anti-DNA Related Idiotypes by IgM from Cord Blood B Cell lines and Clones

## 6.1. INTRODUCTION

Antibodies to deoxyribonucleic acid (anti-DNA), occur prominently in Systemic lupus erythematosus (SLE) and are rarely present in other disease states. Furthermore, it is widely believed that understanding the basis of anti-DNA expression in SLE, would provide a critical insight into the pathogenesis of this disease (Schwartz et al, 1985 and Pisetsky et al, 1987).

I had observed that a high percentage of the cord blood B cell lines and clones I examined, had specificity to DNA, particularly those B cells derived from the CD5 B cell subpopulation (Chapter 4).

Therefore, this study was undertaken, to determine the relationship of anti-DNA antibodies found in disease states, to antibodies expressed in the normal B cell repertoire.

Such a relationship can be investigated in terms of the primary structure of the antibodies themselves and genes that encode them, thus idiotypic studies may yield important clues to the origin of these DNA binding antibodies. This was particularly important in relation to whether or not the DNA autoantibodies found in patients, were derived from the CD5+ or CD5- subset

a) A study was carried out in collaboration with Prof. David Isenberg (UCMSM) and examined cord blood lines for the presence of two anti-DNA related CRI :

i. ***The 16/6 idiotype*** ; first recognised on a human monoclonal anti-DNA antibody form a patient with SLE (Schoenfeld, et al 1983), has been shown to be a common anti-

DNA idiotype, encoded by the human VH germline gene, $V_H26$ (Chen et al, 1988). 16/6 is expressed at a higher frequency by antibodies from SLE patients (Isenberg et al, 1984), and has been reported in other diseases (Konikoff, et al 1987).

ii. ***The PR4 idiotype*** ; was obtained by purifying anti-DNA antibodies from the serum of a patient with leprosy, (Williams et al, 1988) and is also expressed by immunoglobulin from patients with SLE at high frequencies.

In view of reports that a monoclonal paraprotein which reacts with the K30 polysaccharide of Klebsiella, has been shown to react with DNA and carry the 16/6 idiotype (El-Roiey et al, 1987 and Naparstek et al, 1985), IgM from the cord blood B cells, was examined for reactivity against the K30 antigen.

**b)** Further studies were carried out in collaboration with Dr. R. Watts (UCMSM).

***The BEG-2β idiotype*** : In order to investigate further, the nature of DNA binding immunoglobulin, I examined, by means of a direct binding ELISA, IgM from CD5+ and CD5- EBV B cell clones, for the expression of a human fetal DNA idiotype, designated BEG-2 idβ. Monoclonal antibody BEG-2 is a dsDNA binding IgM derived from a human fetus. The BEG-2idβ is present on the μ heavy chain (Watts et al, 1991) and is closely related to a previously reported idiotype (BEG-2idα) on the λ light chain of the BEG-2 molecule (Watts et al, 1989).

It has already been established that BEG-2 idα was present in the serum of both normal individuals and patients

with SLE, TB and RA (Watts et al, 1990 and 1991).

Furthermore, it was suggested that monoclonal antibody BEG-2 shares common idiotypes with murine DNA binding antibodies (Watts et al, 1989), suggesting that autoreactivity may be conserved during evolution.

## 6.2. RESULTS

### 6.2.1. FREQUENCY OF EXPRESSION OF ANTI-DNA ASSOCIATED IDIOTYPES ON CORD BLOOD B CELL LINES.

Using the cord blood lines that had been immortalized with Epstein-Barr virus (EBV) (see Chapter 4), experiments were performed to establish if, anti-idiotype (anti-id) antibodies, prepared against anti-DNA antibodies, can be expressed as part of the normal early V gene repertoire.

#### 6.2.1.1. Frequency of cord blood lines expressing the 16/6 idiotype (16/6 id)

Initial experiments involved examining 70 supernatants, collected from the established EBV cord blood lines.

All the supernatants tested, contained at least 500ng/ml of IgM and were assayed for the expression of the anti-DNA associated 16/6 id using an ELISA. This was kindly performed by Dr. W. Williams (UCMSM) and is described in *Materials and Methods*.

Four of the 70 (6%), EBV immortalized cord blood lines were positive for direct binding to 16/6 id which had been coated onto ELISA test plates. Optical absorbance (OD values) for the 4 positive lines were 0.1-0.2, whereas the remaining lines had OD values of 0.045.

The autoreactivity of the lines examined in this study, are described in Chapter 4 and the 16/6 id was associated in all cases with anti-DNA, anti-Fc and anti-cardiolipin reactivity (Table 6.1).

### 6.2.1.2. Expression of the anti-DNA associated PR4 idiotype by cord blood B cells.

Further examination of the IgM obtained from 70 cord blood B cell lines, investigated the frequency of expression of the PR4 id. This ELISA assay was kindly performed by Dr. W Williams (UCMSM) and is described in *Materials and Methods*.

Supernatants were considered to be positive if the PR4 id level was above the upper limit of a normal range, which was set at the mean +2 standard deviations (SD) of sera from healthy individuals, included in the assay.

However, none of the 70 supernatants examined had IgM which reacted with antibodies to the PR4 id, when compared with the control sera (ELISA results showed OD <0.05, where positive control sera was 1.052 and negative 0.03).

### 6.2.1.3. Specificity for the K30 polysaccharide of Klebsiella by IgM from cord blood cell lines.

A monoclonal paraprotein which reacts with the K30 polysaccharide, has been shown to react with DNA and to carry the 16/6 id (Naparstek et al, 1985).

In order to see any relationship between 16/6 id positivity and K30 specificity, I examined, by ELISA (see Materials and Methods), 4 of the cord blood supernatants, positive for the 16/6 id (section 6.2.1.1, above) and 8 others with anti-DNA activity, for reactivity with the K30 polysaccharide antigen .

Only 1/12 of the supernatants tested, bound to the K30 polysaccharide (OD 0.19). This IgM was not, however, positive for the 16/6 id. (Table 6.1).

**TABLE 6.1 K30 specificity and the presence of the 16/6 idiotype on IgM from cord blood lines**

| SPECIFICITY | | | | | |
|---|---|---|---|---|---|
| **Lines** | **K30** | **ssDNA** | **Fc** | **Cardiolipin** | **16/6** |
| **96** | + | + | + | + | - |
| **83** | - | + | + | - | - |
| **72** | - | + | - | - | - |
| **59** | - | + | + | + | + |
| **88** | - | + | + | + | + |
| **68** | - | + | + | + | + |
| **70** | - | + | + | + | + |

K30 polysaccharide of Klebsiella, detected by ELISA, single-stranded DNA (ssDNA), Fc of IgG, rheumatoid factor, RF (Fc) and cardiolipin reactivity detected by ELISA, as per *Materials and Methods*. 16/6 idiotype assay, kindly performed by Dr. W. Williams, as described in *Materials and Methods*.

## 6.2.2. EXPRESSION OF AN ANTI-DNA ASSOCIATED IDIOTYPE ON IgM from CD5+ AND CD5- CORD BLOOD CLONES.

Having established that the 16/6 id, was part of the normal repertoire early in life, it was of interest to determine if there was a correlation with the expression of anti-DNA associated idiotypes and early B cell CD5 positivity.

I examined IgM from the CD5+ and CD5- derived cord blood clones for the expression of an anti-DNA associated id, which had been identified within our laboratory and designated BEG-2idβ (Watts et al, 1990). The BEG-2 antibody allowed an investigation of the expression of a human fetal monoclonal, DNA binding antibody in relation to early CD5+ and CD5- B cells.

### 6.2.2.1 Cord blood B cells expressing the Beg2β idiotype.

We analysed the expression of the human fetal DNA id, Beg2 idβ, using a polyclonal rabbit anti-idiotype antiserum, and a direct binding ELISA as a screening assay. The data was analysed using a value of x5 the median optical density values, or greater to indicate positivity. ( see *Materials and Methods*).

64 culture supernatants containing monoclonal antibody, derived from CD5+ and CD5- cord blood B cells, were investigated for the presence of the BEG-2 idβ, Fig.6.1.

BEG-2 idβ was found to be present on 3% (2/64) of the antibodies obtained from all the clones in the study. Thirty-three (51%) of these had reactivity with ssDNA.

The 2 supernatants that gave a positive result (derived from clones designated 236 and 56 ; see section

6.2.2.2) were titred out and dose-dependent binding curves are shown in Fig.6.2.

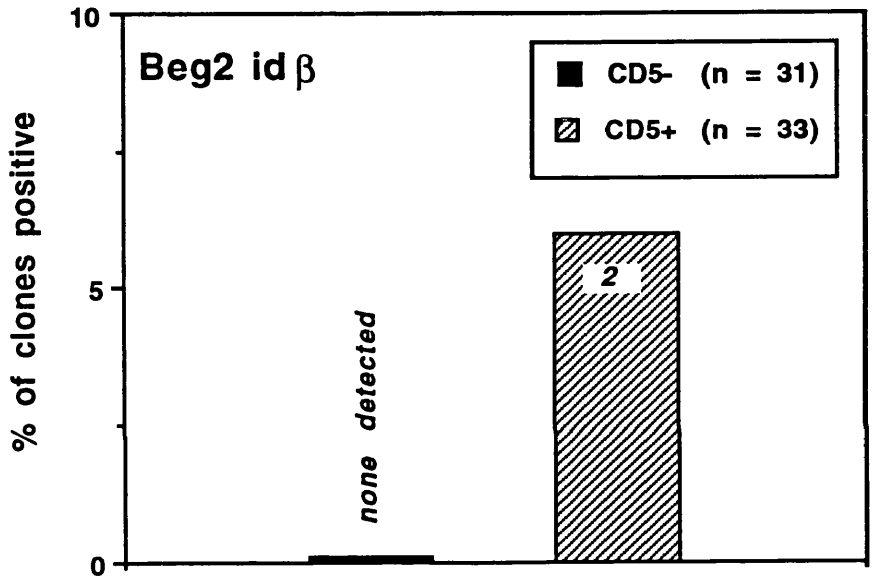


**FIGURE 6.1 EXPRESSION OF BEG 2 IDβ BY IgM FROM CD5+ AND CD5- CORD BLOOD B CELLS.** The frequency of expression of the Beg 2 idβ by 31 CD5- and 33 CD5+ cord blood EBV B cell clones, is expressed as the percentage of clones positive and represented as histograms for each group. Positivity was assessed by direct binding ELISA and calculated from optical density (OD) values, expressed as multiples of x5, or greater, of the median of the samples tested in each assay (see *Materials and Methods*).

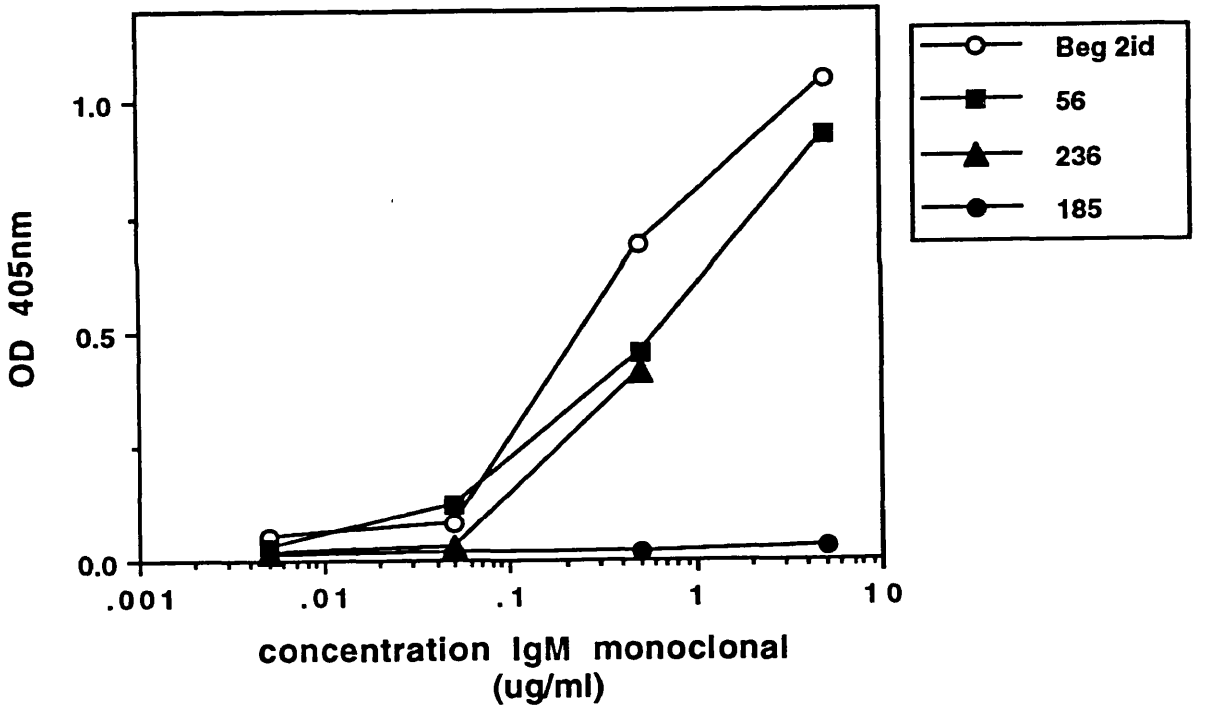


**FIGURE 6.2. BINDING OF IgM FROM CORD BLOOD B CELL CLONES TO RABBIT ANTI-ID BEG-2β.** Dose-dependent binding of cord blood clones, designated 236 ,56 and 185 to solid-phase anti-id BEG-2β. The binding activity of the IgM from each group is expressed as optical absorbance (OD) at 405nm. Monoclonal antibody, 185 does not bear the Beg-2 idβ. Direct binding of BEG2 idβ to rabbit anti-id BEG-2β is shown.

To formally confirm the presence of the BEG-2 idβ on the IgM molecules, found to be id positive by direct binding assays, immunostaining of a Western blot, following SDS-PAGE of denatured monoclonal antibodies, 56 and 236, were performed. IgM from an id negative cell line, 152, was included as a control.

One track from each blot was stained to identify human IgM, kappa light chain, lambda light chain and BEG-2idβ.

Fig.6.3 shows the presence of the idiotype on the IgM blotted from clone 56, expressing kappa light chains and not on the id negative IgM ( cell line, 152).


**FIGURE 6.3. PRESENCE OF BEG-2 IDβ ON IgM FROM CORD BLOOD B CELLS.** The IgM preparations from clone 56 and cell line, 152, were run on a 10% SDS-PAGE and electroblotted (as per Materials and Methods).

Western blots of Lanes 2-5 : 56
Lanes 6-9 : 152

stained with Lanes 2 and 6 : anti-human IgM
Lanes 3 and 7 : anti-human kappa
Lanes 4 and 8 : anti-human lambda
Lanes 5 and 9 : anti BEG-2 idβ

### 6.2.2.2. Reactivity of the BEG-2idβ positive B cells.

The source, isotype and binding specificity of the BEG-2idβ positive monoclonals, in the panel of CD5+ and CD5- cord blood B cell clones examined, is shown in Table 6.2.

Interestingly, the 2 cord blood derived antibodies with BEG-2 idβ positivity, were derived from the CD5+ population of B lymphocytes. That is, 2/33 (6%) of CD5 clones carry the BEG-2β id (Fig 6.1). None of the 31 CD5- clones were BEG-2β positive.

The clones of B cells producing the id positive IgM, designated 236 and 56 express λ and κ light chains, respectively. Data obtained in Chapter 5, revealed them to both belong to the VHIV family of genes.

Furthermore, both monoclonals are associated with ssDNA binding and, in fact, are polyreactive, also binding to collagen and in the case of clone 56, to vimentin and cardiolipin.

**TABLE 6.2 Characteristics of the BEG-2 idβ positive cord blood clones**

| MONOCLONAL ANTIBODY | SOURCE | ISOTYPE | SPECIFICITY | VARIABLE REGION FAMILIES | | |
|---|---|---|---|---|---|---|
| | | | | VH | Vκ | Vλ |
| 236 | CD5+ cord blood | IgMλ | ssDNA, collagen | IV | - | NK |
| 56 | CD5+ cord blood | IgMκ | ssDNA, RF, collagen, vimentin, cardiolipin | IV | III | - |
| Beg2 | Fetal liver | IgMλ | ssDNA, human serum albumin | IV | - | NK |

ssDNA ; single-stranded DNA, RF ; rheumatoid factor, NK ; not known

### 6.2.2.3. Blocking of binding of BEG-2idβ positive antibodies to DNA by anti-id Beg2β

Since BEG-2 idβ positivity among the cord blood B cells, was found to be associated with DNA binding, it was demonstrated that incubation with rabbit anti-id BEG-2β was able to inhibit the binding of the id positive monoclonal antibodies, 236 and 56, to ssDNA (as described in Materials and Methods), shown in Fig.6.4.

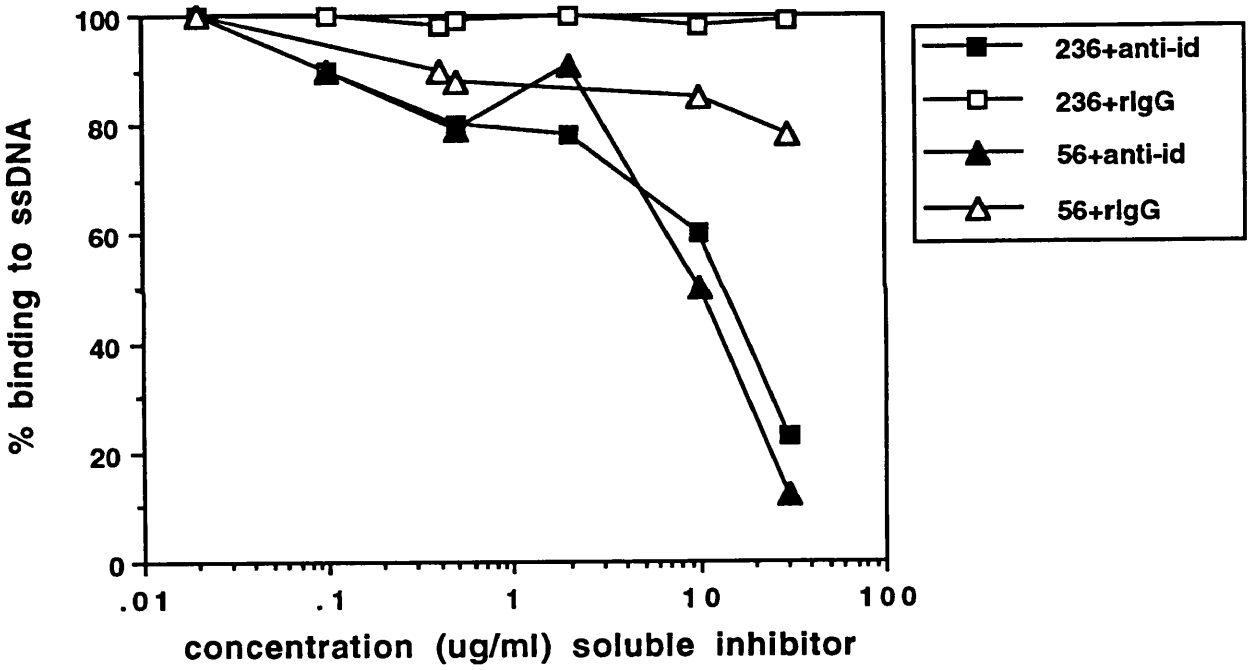


**FIGURE 6.4 INHIBITION OF BINDING OF CORD BLOOD ANTIBODIES TO DNA BY ANTI-IDIOTYPE (ID) BEG2β.** Dose-dependent inhibition of monoclonal IgM from cord blood clones 56 and 236 to solid-phase ssDNA by soluble anti-id BEG-2β. The binding observed in the presence of the soluble inhibitor (anti-id) is expressed as a percentage of the binding activity measured after incubation of the IgM, under identical conditions,but in the absence of any soluble inhibitor (anti-id) (100% binding activity). rIgG, rabbit IgG, with which no significant inhibition was observed.

## 6.3 DISCUSSION

In studies, previously described (Chapter 4), I have shown that both CD5+ and CD5- B cells within the early B cell pool are programmed to recognise one or many self antigens. Many of the specificities include anti-DNA and are not confined to the CD5 B cell subset.

In the studies, presented in this chapter, I further examined the nature of these immunoglobulins, by analysing their expression of human DNA idiotypes (id).

Others have shown that anti-DNA idiotypes occur, commonly, in the sera of patients with SLE, or animal models of the disease, characterised by the production of autoantibodies against a wide range of autoantigens, including DNA (Tan et al, 1989, Rauch et al, 1982, Datta et al, 1983, 1986, Mayus et al, 1985 and Halpern et al, 1985). Although such autoantibodies are usually associated with disease, natural antibodies against some of these antigens, especially DNA, may be detected in the serum of normal individuals (Guilbert, Dighiero and Avrameas, 1982).

My studies illustrate the relationship of anti-DNA to normally expressed antibodies :

**a) 16/6 id expression by IgM from cord blood B cells**

EBV transformed cord blood B cells, express the anti-DNA associated CRI, designated 16/6, which is often associated with autoantibodies in SLE. (Schoenfeld et al, 1986). Interestingly, the DNA binding antibodies, carrying the 16/6 id are encoded by the conserved $V_H26$ germline gene (Chen et al, 1988), therefore the data supports the notion that antibody genes coding for anti-DNA are present as part of the

normal early V gene repertoire. Similarily, in autoimmune strains of mice, it has been shown, that a gene from a DNA binding antibody is identical to a VH gene in normal mice (Treppicchio et al, 1987).

In all cases examined, the 16/6 id was associated with antibodies with specificity for DNA, among other self-constituents. Furthermore, one of the cord blood cell lines tested had Klebsiella, K30 specificity.

It has been proposed that autoantibodies, are in fact, anti-ids to anti-viral antibodies (Plotz et al, 1983). As such, they carry the internal image of the viral antigen and can therefore bind to the virus receptors on cells.

Such a connection exists between RF and anti-bacterial antibodies (Johnson et al, 1985). Immunization with human RF, induces antibodies reactive with streptococcal peptidoglycan polysaccharide (SSP) antigen and RF. Thus, RF is an anti-id to anti-SSP antibodies or carries an internal image of the antigen epitope.

Such an idiotypic relationship exists between anti-DNA antibodies, bearing the 16/6 id and antibodies to Klebsiella. The human monoclonal IgM anti-DNA antibody, 16/6, was generated from a patient with SLE and correlates with the activity of the disease (Shoenfeld et al, 1983). Subsequently, it was found in IgM immunoglobulins in normals. It was realised that patients with Klebsiella infections have increased incidence of high titres of the 16/6 id, compared to control groups. Both the 16/6 id and anti-Klebsiella antibodies can be absorbed by the Klebsiella K30 antigen (El-Roiey et al, 1987). The implication being, that the 16/6 id is present on antibodies binding Klebsiella antigens in non-autoimmune

subjects and on autoantibodies to DNA in patients with lupus.

However, in the studies we performed, there was no association between 16/6 positivity and the Klebsiella antigen. It would be interesting to investigate the cord blood B cell response to the K30 antigen *in vitro* .

The lack of association of K30 with 16/6 in the B cell examined, could indicate that K30 binding is not related to the $V_H26$ germline gene. However, other factors may be involved. For example, the light chain may be important. Interesting experiments showed that a paraprotein with specificity for the Klebsiella K30 polysaccharide, has striking sequence homology with the $V_\kappa I$ light chains (Atkinson et al, 1985) and antibodies that have been sequenced, have shown that, those carrying the 16/6 id, utilize $V_\kappa I$ light chains. It may be significant that a high number of the EBV clones in my studies, utilize $V_\kappa III$ light chains.

My results, do, however, confirm the finding, of an association of 16/6 id with DNA specificity, in normal IgM and that seen in autoantibodies of patients with disease (Isenberg et al, 1985).

In contrast to this, the PR4 id does not appear to be present on cord blood B cells, at least at a frequency detectable with the number of clones I examined. This may be interpreted as suggesting, perhaps, an independent selection of these CRI (16/6 and PR4). Cross-inhibition studies have in fact, demonstrated that 16/6 id and PR4 id, although both identified as common anti-DNA idiotypes, detectable in the serum of many lupus patients, are not identical (Lockniskar et al, 1984).

**b) Expression of BEG-2idβ by IgM from CD5 B cells**

The relationship of the normal expression of anti-DNA associated CRI and CD5 expression, was not clear, from the studies described above. Since the 16/6 id was no longer available for my use, I identified the expression of an idiotype, isolated from a human fetal DNA binding antibody, by IgM from the panel of CD5+ and CD5- derived cord blood clones I had established (Chapter 4).

Designated BEG-2idβ, this idiotype is located on the μ heavy chain of the monoclonal antibody BEG-2 molecule (Watts et al, 1991).

Interestingly, those clones that were BEG-2idβ positive, were originally obtained from the CD5 sorted cells, of which a significantly higher number of clones had been shown to produce anti-DNA antibodies. Furthermore, the binding of the BEG-2idβ positive antibodies to DNA, was inhibited by incubation with anti-id BEG-2β, suggesting that the idiotype may be located on or near the DNA binding site.

Other groups (Hillson et al, 1990), analysed the expression of BEG-2idβ on fetal and adult antibodies, and observed a smaller percentage of fetal antibodies, than adult, that were id positive. Further, the adult antibodies expressed a smaller percentage of BEG2idβ positivity than was observed for my cord blood clones. It would appear, therefore, that there is a trend towards the BEG-2idβ becoming less commonly expressed, as the immune system matures, perhaps indicated by the loss of the CD5 phenotype. In relation to this, it is interesting that, recent data, reported the presence of the BEG-2idβ, in the serum of patients with RA and SS, but not SLE (Watts et al, 1991). My studies (Chapter 3), and those of others

(Plater-Zyberk et al, 1986 and 1989), have reported increased percentages of CD5 B cells in RA and SS.

These results, may reflect a preferential expansion of B cells, likely CD5 and reflect a unique distribution of this id among human B cell subsets.

The BEG-2 antibody was shown to be idiotypically related to several murine DNA binding antibodies (Watts et al, 1990), indicating a conservation of these autoantibodies through evolution.

In fact, it was recently shown that monoclonal antibody, BEG-2, is encoded by a VHIV gene, determined by nucleotide sequencing (Brown et al, 1990). However, the BEG-2idβ is likely, only present on a subset of VHIV antibodies, as many of the cord blood clones examined expressed VHIV family genes, but were id negative. However, both of the CD5+ clones, that were BEG2idβ positive, expressed VHIV family genes, as determined by the monoclonal antibody, Lc1 (as described in Chapter 5). The Lc1 antibody is probably a marker for proteins encoded by the 71-2 and 71-4 and related genes, belonging to the VHIV family (Kodaira et al, 1986). My findings were confirmed by Watts et al, 1990, who demonstrated that, among the reported germline genes, VH71-2 (Lee et al, 1987) is that most closely related to VHBEG-2.

Furthermore, the BEG-2idβ is not limited to VHIV related antibodies and appears to recognise a set of antibodies, encoded by VHVI genes (Watts et al, 1991). It has, however, been shown that murine idiotypes, may be encoded by VH genes from different families (Schlomchik et al, 1990).

The CD5 cord blood clones, that expressed the BEG-2β id, did so in association with kappa and lambda light chains. It

seems likely, therefore, that the expression of the id does not rely on a contribution from light chains, in the whole molecule. It may be that, binding of the anti-id, may be related to charge. This would affect the recognition of the antibody on different (structurally) molecules.

When the deduced amino acid sequences of the BEG-2 heavy cahin (encoded by VHIV), were compared to another id positive protein, but encoded by VHVI, the sequences were similar in regions in the framework (FR), FR1 and FR3 areas (Watts et al, 1991). This related positions of cationic lysine and arginine residues, thought to play a role in the binding of anionic antigens, such as DNA (Nelson et al, 1985 and Schlomchik et al, 1987). Many of these are found outside the classical binding site. Homology in these regions may or may not be sufficient to create the idiotype and indeed similar DNA binding proteins may be id negative.

Furthermore, this may have consequences in disease states, whereby an antibody's ability to participate in immune complex disease, is dependent on properties such as avidity, isotype and ability to fix complement. There is evidence that the charge of an antibody (as well as the antigen), within an immune complex, influences the site of tissue deposition (Ebling et al, 1980). The charge of an antibody, may also help explain the contribution of polyreactive autoantibodies to the formation of circulating immune complexes, present in the sera of lupus patients (Louzir et al, 1988).

Finally, both the BEG-2idβ positive cord blood clones in my study, were polyreactive and had the ability to bind dissimilar antigens, including binding to Fc of IgG, collagen, vimentin and cardiolipin, as well as to DNA. Polyspecificity may suggest

that the antigens bound by these antibodies, have regions of close structural homology. Indeed, anti-DNA production may be the result of stimulation by a cross-reactive antigen, either self or foreign.

In conclusion, the data suggests that DNA associated CRI, are not exclusively found on anti-DNA antibodies, but can be present on autoantibodies of other specificities. Since any representation of an antibody sequence in the germline configuration, suggests a conserved, possibly protective function for that antibody, it would seem that the ability to express anti-DNA antibody is extremely beneficial.

# Chapter 7

# GENERAL DISCUSSION and CONCLUSIONS

This thesis was concerned with characterising the subpopulation of human B lymphocytes, expressing a 67KDa T cell marker and known as CD5 (B-1) B cells.

An investigation of the antibody producing potential of human cord blood B cells, and in particular, an evaluation of their frequency of $V_H$ and $V_L$ gene utilization, has occupied the main part of the study.

A number of significant questions arise, while interpreting the observations made in these studies and ways in which to further elucidate the mechanisms of action of CD5 B cells, become apparent. These are outlined and discussed below, and, based on my conclusions, I propose a model for the possible development and function of human CD5 B cells :

## 7.1 WHAT IS THE SIGNIFICANCE OF POLYREACTIVE AUTOANTIBODIES TO CD5 B CELLS ?

The high frequency of auto and polyreactivity, within both the CD5+ and CD5- cord blood B cell subsets, suggests that such specificities are a normal part of the human B cell repertoire, and must, therefore, be important.

In particular, the role of IgM anti-Fc of IgG (rheumatoid factor, RF) and IgM anti-ssDNA antibodies, as evidence of *natural* autoimmunity, should be considered.

### a) The molecular basis of reactivity (connectivity)

Lymphocytes can recognise structures on antigen receptors, produced by or present on other lymphocytes. The response of these so-called idiotypic structures may result in the formation of anti-idiotypic antibodies and the construction

of a formal network of idiotype-anti-idiotype interaction (Jerne, 1974). The co-existance of sets of complementary idiotypes and anti-idiotypes, has been established in a variety of systems (Kelsoe and Cerny, 1979, Pollock et al, 1983 and Schrater et al, 1979), and such idiotypic regulation may apply to the process of anti-self responses.

However, it has yet to be formally established if polyreactive antibodies, display connectivity, ie, interclonal idiotypic cross-reactivity, as has been suggested for the mouse (Vakil and Kearney, 1987). A restricted use of germline IgV genes, by polyreactive antibodies would suggest this. In fact, polyreactive IgM, is highly represented in the sera of various fish species, of very distant phylogenetic orders, for example, sharks, torpedoes, salmon (Gonzalez et al, 1988) and must, therefore, be encoded by restricted families of germline genes. Indeed, recent studies have shown that human (Sanz et al, 1989) and mouse (Baccala et al, 1989) polyreactive/natural antibodies, are encoded by germline genes, without or with very few mutations.

The molecular basis underlying the behaviour of multispecific antibodies, relies on the notion that their antigen binding sites are considerably greater, than that needed to accomodate only a single epitope. Furthermore, they are complementary to a number of different ligands (Richards et al, 1975 and Amit et al, 1986).

Comparison of amino acid sequence data of murine light chains (Schlomchik et al, 1986), has introduced the idea that framework rather than complementarity determining regions may confer RF activity. Therefore, unconventional binding sites may contribute to multispecificity and may

explain why crossblocking can sometimes be demonstrated.

The structure(s) recognized by multispecific antibodies, on the various antigens is/are not known, but murine studies on natural antibodies suggest that polar amino acids play an important role in the interaction of these polyreactive antibodies (mainly arginine and lysine) with the various antigens (mainly glutamic and aspartic acid) (Avrameas et al, 1989). Polyspecificity may suggest that the antigens bound by these antibodies have regions of close structural homology, despite having very different chemical compositions.

Alternatively, these antigens may contact the antibody surface at different sites, thereby offering an explanation for their ability to bind dissimilar antigens (Lafer et al, 1981, Rubin et al, 1984 and Jacob et al, 1984).

**b) Potential functions of polyreactive antibodies**

The functional significance of multispecific antibodies is unclear at present. They might be products of cells destined to be triggered by exogenous antigens and to produce, after somatic mutations, antibodies with greater specificity towards the inducing antigen (Ternyck and Avrameas, 1986 and Naparstek et al, 1986). Alternatively, they might be transporters of catabolic products, (Grabar, 1983) or play a role in the establishment of the B cell repertoire (Kearney and Vakil, 1986). It may be that they are evolutionarily destined to bind to a variety of antigens, and so provide a first line of defence in an immune response, either during ontogeny, or to generate a specific response.

Polyreactive natural antibodies are able to interact

with both self and non-self antigens (reviewed by Avrameas, 1988). Polyreactive IgM, owing to its nature and multivalency, will aggregate bacteria and viruses and damaged self constituents, mediate complement dependent lysis and encourage and enhance phagocytosis and opsonisation (Cross et al, 1989, Narin et al, 1989, Matsiota et al, 1989, Gonzalez et al, 1989, Lutz et al, 1987 and Hinter et al, 1987).

Furthermore, polyreactive antibodies may initiate an immune response. RF activity may up-regulate the actions of specific IgG, aggregated as a result of binding to the surfaces of bacteria and viruses (see below).

*i IgM RF autoantibodies*

IgM RF autoantibodies are probably not pathogenic. One possibility, suggested by Cohen and Cooke, is that they serve to mask autoantigenic epitopes from the immune system, thereby preventing the triggering of potentially more damaging, high affinity IgG autoantibodies.

It is possible that RF can amplify the humoral immune response to certain microrganisms (Goni et al, 1983). Although multimeric IgM RF reacts weakly with monomeric IgG, the RF molecule may bind tightly to IgG aligned on a solid surface, such as a bacterial cell wall. For this reason, IgM RF can stabilise the binding of low affinity IgG antibodies, that are produced during an early polyclonal immune response to an infectious agent. The IgM RF also promotes the clearance of immune complexes, which would be particularly important for those IgG subclasses that don't readily fix complement.

It has recently been postulated that the major role of

RF B cells is independent of antibody secretion, but rather relates to antigen processing (Carson et al, 1991). B cells making autoantibodies, are very efficient presenters of antigen to T cells and recently it has been shown that B cells with RF activity, can effectively process antigen of any form, if it is complexed with IgG antibodies (Roosnek and Lanzavecchia, 1991). Furthermore, with increasing complex formation with IgG, in a situation of excess antibody, B cells could be inhibited by their Fc receptors (Cambier et al, 1989).

It was recently observed that RF precursors (17.109 positive cells), are abundant in the mantle zones of lymph node and tonsil, interestingly, often where immune complexes localize (Maclennan et al, 1982). Thus, RF precursor cells may be important for the processing of antigen trapped in immune complexes. Carson et al, 1991, proposed that, by such a mechanism, spontaneously arising autoantibody-autoantigen complexes, would be captured by RF precursors in the mantle zone and processed and presented as self-peptides to autoreactive T cells. The release of a T cell inhibitory cytokine, would prevent a B cell clonal expansion. Since autoantibodies, because of somatic mutation of Ig genes at random, probably appear throughout life, this mechanism would prevent the expansion of autoreactive T and B cells.

In view of my observations, of a higher frequency of cord blood CD5 B cells, that are 17.109 positive, (the phenotypic marker of the Vκ325 germline gene (Chen et al, 1988)), it would appear that, V genes, that encode both CRI and autoantibodies, may be preferentially expressed by this subset of B cells, for the purposes described above.

The B-1/CD5 B lymphocyte population seems a likely

candidate, to be a cellular source, of the RF that is regularly found in conjunction with polyclonal B cell activation, particularly at the sites of microbial infection. Interestingly, preliminary data has established, specificity for the 65kD mycobacterial antigen among the cord blood CD5+ separated B cell clones (data not shown). Experiments also carried out in our laboratory (Lydyard et al, 1990), showed that, a number of cord blood B cell clones produced IgM which reacted with microrganisms, and it is well established that murine CD5 B cells secrete IgM antibodies against bacterial polysaccharides as well as autoantibodies (Hayakawa et al, 1984). Its not resolved if this type of reactivity is a property of interaction between the antigen and the IgV region or between the antigen and sugar molecules on the immunoglobulin structure, it will, however, focus antigen to initiate germinal centre formation.

*ii DNA autoantibodies*

Antibodies to DNA occur prominently in SLE, and although the expression of serum anti-dsDNA antibodies, is essentially exclusive to SLE, B cells capable of producing anti-ssDNA antibodies, occur with high frequency in normals as well as patients with the disease (reviewed by Pisetsky, 1988).

My data revealed a high frequency of autoreactive fetal/neonatal B cells with specificity for DNA. However, I also found, that, in addition to reacting with DNA itself, many of the antibodies also have activity with IgG, collagen, mitochondria, perinuclear and cardiolipin antigens, to varying degrees (Chapter 4).

The fine specificity of DNA binding has been

researched in normals and individuals with disease (Pisetsky et al, 1984, Andrzejewski et al, 1981, Shoenfeld et al, 1983 and Cairns et al, 1984) and there appears to be considerable variation in the pattern of binding of antibodies to DNA in terms of their specificity and avidity, probably related to the structural heterogeneity of DNA itself, mitochondria, perinuclear and cardiolipin antigens, to varying degrees (Chapter 2). Studies on the properties of anti-DNA producing B cells are therefore of great interest.

My data, indicated that a higher frequency of CD5 B cells had specificity for DNA in the early B cell pool. Furthermore, others have also shown that, in both man and mouse, the population containing anti-DNA B cells, is the Leu1+ (Hayakawa et al, 1984) and Ly1+ (Casali et al, 1987) subpopulation, respectively. If these cells respond more readily to activation signals (Mayus et al, 1985), then non-specific stimulation could lead to their preferential expression and expansion.

Since polyspecific anti-DNA antibodies seem to be conserved among species, they must be beneficial to an organism. They may in fact be cross-reactive with a self or foreign antigen. Recent investigations suggest an unusual candidate for a molecule inducing cross-reactive anti-DNA antibodies (Karounos et al, 1988). It was shown that, normal sera, despite having high titres of antibodies to DNA from two bacteria, Micrococcus lysodeckticus and Staphylococcal epidermidis, had low titres of antibodies to DNA from mammalian sources and other bacteria (for example, E. Coli). The antibodies to micrococcal and staphylococcal DNA are highly specific to antigenic sites on the molecules, in contrast

to SLE anti-DNA antibodies which react to all types of DNA. The latter clearly recognise a commonly expressed determinant and it would appear that unique sequences or configurations could make DNA immunogenic and induce an immune response.

Idiotypic networks may be important in the control of immune responses to autoantigens. For example, anti-idiotypes to DNA specific autoantibodies have been described in the sera of patients with inactive SLE which were absent in those with active disease. This would suggest that the presence of these antibodies may influence the course of a disease (Shoenfeld et al, 1989).

One should bear in mind, that laboratory assays, on the specificity of polyreactive antibodies, are complicated by the inherent structural diversity of antigens such as DNA, as well as, for example, the difficulty in obtaining preparations free of contaminating molecules, ie, ssDNA not contaminated with dsDNA and *vice versa*. Assays may differ in their sources of autoantigens (mammalian, bacterial) and so vary in their ability to recognise autoantigens, with varying conformational properties. Indeed, the manner in which antibody binding is detected may differ, thus, affecting the strength of binding detected.

In conclusion, although the features which confer polyspecificity are uncertain, they appear to occur commonly among B cells. Furthermore, in the case of cord blood B cells, such immunoglobulin, with features similar to those of natural antibodies, are produced, not only by the CD5+ B cell subset, but also by some CD5- B lymphocytes.

## 7.2 IS THERE A FUNDAMENTAL DIFFERENCE BETWEEN ADULT AND CORD BLOOD CD5 B CELLS ?

My results would suggest that CD5- B cells, in cord blood, clearly have similar properties to CD5+ B cells in cord blood and to those reported for CD5 B cells in adults (Casali et al, 1987).

A number of interpretations of these findings, may be made :

**a) Possible differences between B cells expressing surface CD5 and those that do not.**

*i.* CD5 expression on B cells may be modulated within the fetal microenvironment. In fact, it was recently shown that CD5 expression was modulated as B cells moved through the cell cycle. These changes in surface CD5 were not due to the regulation of mRNA levels (Brooks et al, 1992). Furthermore, data from our laboratory, has shown that CD5- cord blood EBV B cells can express mRNA for CD5 (Paavonen et al, 1990). It would appear therefore, that other factors are involved, and CD5- B cells may constitute an activation and/or proliferation stage of CD5+ B cells, associated with the loss of the surface CD5 molecule.

*ii.* The adult B cell repertoire may contain many CD5- B cells with high affinity surface immunoglobulin receptors, resulting from continuous exposure to environmental antigens. The adult CD5+ population, however, may be equivalent to the majority of cord blood B cells which have not been exposed to such antigens.

Most of the cord blood B cells are CD5+ and at least

some may be "mutating" away from polyreactivity to more restricted specificity (discussed below).

*iii.* An interesting possibility is that many CD5- cord blood B cells have characteristics of the CD5- "sister" population, described in the murine peritoneal cavity. Three B cell lineages were postulated : conventional B cells (CD5-), B-1a (CD5+ Ly1B) B cells and B-1b (CD5- Ly1B"sister") cells (Herzenberg et al, 1986, 1992 and Kantor et al, 1992). The progenitors that give rise to conventional B cells function normally throughout life. However, the progenitors that give rise to B-1 cells, cease to function after a critical number of B-1 cells develop or are introduced into an animal. Such a self-replenishing mechanism is well known for avian B cells, which develop in the bursa during the first weeks of life. Therefore, in a developmental sense, B-1 cells appear to be more "primative" than the conventional murine B cells.

Indeed, a discrete CD5- B cell subset, has similarily been observed in man. This B cell subset was segregated on the basis of its expression of low levels of CD45RA, the high MW isoform of the leukocyte common antigen (L-CA, B220, T200), and had many of the characteristics of CD5 B cells, including the production of autoantibodies, (Kasaian et al, 1992).

**b) Possible major differences in the fetal and adult B cell pool**

It may be misleading to compare results obtained with adult CD5 B cells (Casali et al, 1987, Hardy et al, 1987 and Sanz et al, 1989) and the cord blood CD5 B cells in this study, for the following reasons :

*i.* CD5 B cells represent a considerable proportion of neonatal spleen (Hayakawa et al,1987 and Hardy et al, 1987) and analysis of the neonatal response to antigen (Neil and Klinman et al, 1982) and of neonatal cell surface markers (Kinkade et al, 1984), led to the conclusions that B cells in the fetal liver and early after birth in the spleen, represent a distinct B cell lineage from that of the adult B cell pool.

One of the major differences in B cells emerging in late fetal and early neonatal stages, compared with B cells emerging from the bone marrow in adults, concerns the timing of sIg expression. Adult bone marrow cells do not divide very much after light chain rearrangement and sIg expression (Osmond et al, 1986), whereas a clonal development in Balb/c neonatal mice continues for several days after these events (Klinman et al, 1978). Therefore the neonatal and also, CD5 B cell repertoire may be distinct, in that B cells developing from bone marrow may not pass through the same sequential acquisition of variable region specificities.

*ii.* In the mouse, studies of Abelson virus transformed pre-B cell lines (Yancopoulos et al, 1984 and Lanier et al, 1987) and fetal liver hybridomas (Perlmutter et al, 1985), clearly demonstrate the majority of fetal transcripts to be encoded by members of the VH7183 gene family, particularly the VH81X gene. Remarkably, human and murine VH sequences are closely related (Lee et al, 1987) and this particular gene is homologous to the 51P1 gene segment which belongs to the human VHIII family and is frequently expressed during early development (Chen et al, 1990). Indeed, my studies revealed a higher frequency of usage of VHIII family genes by cord blood B

cells.

It is interesting that the structural homology found between murine and human VH sequences, is concentrated in variable regions, not normally associated with antigen binding (Rechari et al, 1983). The resulting antibodies could originate from CD5 B cells and may not in fact be participating in the classical role of guarding against infection.

It has been shown that the most JH proximal VHVI gene family in humans is frequently expressed early in life (Logtenberg et al, 1989). Evidently, from my data and from others, other VH families are also featured in ontogeny (Perlmutter et al, 1989).

It is assumed that a programmed heavy chain gene rearrangement takes place and due to the chromosomal order of gene segments involved, ie, VH gene segments appear to preferentially rearrange in relationship to their proximity to JH, (Perlmutter et al, 1985 and Yancopoulos et al, 1984). Since human VH gene segments appear to be positioned in family independent clusters (Kodaira et al, 1986), it is still possible that it may be the position on the chromosome that determines the rearrangement timing during B cell maturation.

Ultimately, a mechanism must exist, whereby only B lineage cells expressing H chains, likely to be useful, are selected for expansion.

*iii.* Finally, environmental influences will have little impact on these early cells, so you might expect them to have different specificities anyway. CD5 B cells are only produced in a "window" from birth to 2 months (Lalor et al, 1989) and studies on mice repopulated with CD5 B cells have shown poor

responses to T dependent antigenic stimulation and a low CD5 B cell response to standard haptenic determinents such as NP (Forster and Rajewsky, 1987). On the other hand, these cells appear to give rise to substantial amounts of anti-phosphoryl choline (PC) antibodies and anti-dextran antibodies via T cell independent stimulation. Since these antibodies are similar to those derived from the bone marrow and splenic cells, it is likely that at least some aspects of repertoire expression in CD5 B cells and conventional B cells overlap.

Indeed, immunologically defective CBA/N mice, ie, they have a low level of PC responsive B cells, may be a reflection of the scarcity of CD5 B cells to be found in these animals.

In conclusion, CD5 B cells are known to exist at different stages of activation, proliferation and secretion, including malignant stages. Therefore, general assumptions cannot be drawn about CD5 B cells, by examining only certain samples of lymphocytes, as I have done in my studies.

Based on the observations discussed above, my data must be interpreted, strictly within the framework of what is known,of the developmental pattern of the B cell lineage, ie, the CD5 B cells, I examined, are a "frozen" population and may be similar to their predecessors or the B cells they give rise to, but may have lost or gained some characteristics.

It is arguably misleading to claim CD5 B cells are immature B cells as there is no proof they will ever mature to be "classical" B cells, or indeed that they will not !

## 7.3 ARE CD5 B CELLS SELECTED BECAUSE OF THEIR IMMUNOGLOBULIN PHENOTYPE OR FOR THEIR ANTIGENIC REACTIVITY?

I used a number of monoclonal antibodies, that recognise subgroup specific epitopes and cross-reactive idiotopes (CRI) associated with individual VH and VL gene family products, to examine IgV gene usage in CD5+ and CD5- B lymphocytes. Although I found specific usage of a VHIV subfamily, and a bias towards use of the Vκ325 germline gene, by CD5 B cells, auto and polyreactivity did not appear to be the property of any particular V gene repertoire.

Furthermore, although, in these studies, the expression of these CRI was found on immunoglobulin with different specificities, their associations with different V gene families was not. This confirmed previous findings, the G6 and G8 CRI and B6 and D12 CRI have always been found to be associated with VHI and VHIII gene families respectively (Mageed et al, 1986, 1990, Crowley et al, 1988, 1990 and Kipps et al, 1989). VHI associated CRI are strongly implicated with RF specificity (Crowley et al, 1988 and Shokri et al, 1990), although VHIII associated CRI have no significant association with this, in monoclonal RF (Crowley et al, 1990), or indeed in the studies presented here.
Therefore :

**a) Does the culture system affect the outcome ?**

Initially, one must consider the extent to which the experimental system I employed in these studies, affected my results :

*i*. The bias, observed, towards the increased frequency of

17.109 positive B cells, may merely be a reflection of the significantly increased numbers of kappa light chain bearing clones, I found, in the CD5 B cell population. Similarily, there may be a trivial explanation for the exclusive representation of 9G4 positive B cells by the CD5+ population, in that, there was simply a statistical problem and insufficient CD5- B cells were examined.

*ii.* Ultimately, the influence of EBV must be considered. This may be significant in light of my observation that G6 was not detectable on any of the cord blood clones generated in the study. There are a number of possible explanations :

a) The cells expressing G6, may be derived from a population at a different stage of differentiation. There is evidence that EBV preferentially stimulates B cells early in their differentiation pathway (Silverman et al, 1987).

b) The cells may have been activated but not "trully" immortalized.

c) There may be a polymorphism amongst individuals expressing this CRI (as discussed in Chapter 5).

d) Perhaps integration with EBV, leads to mutation away from the germline. Possibly different activators have differing effects, and this would be interesting to pursue. For example, SAC induced polyclonal B cell activation is highly selective for $V_H III\mu$ B cells, (Humphries et al, 1988), due principally to SpA binding to the Fab region of immunoglobulin on the cell surface (Romagani et al, 1982).

Preliminary experiments I performed, have shown that lower levels of G6 can be detected in native cord blood B cells,

6 weeks after culture with EBV. This would argue that G6 positive B cells were originally induced to proliferate in response to EBV, but that, these cells could have been disadvantaged by the growth of other cells. Equally, this CRI may require other microenvironmental influences, not present in these cultures, in order to be expressed at high levels.

However, the report that IgM EBV committed B cells are derived from a population of large activated lymphocytes (Chan et al, 1986), and the observations that cord blood B cells express the activation markers CD78 and CD23 (my observations and Hannet et al, 1992), would suggest that EBV does not trigger a restricted repertoire in my system. Furthermore, EBV transformed B cells derived from fetal blood have been shown to have a VH family usage, similar to that of an untransformed population of cells (Guigou et al, 1991).

**b) Is there a reason for the expansion of selected idiotopes on B cells ?**

The persistent expression of CRI may represent regulatory idiotypes, shared by antibodies with different specificities and encoded by various variable region gene families. Furthermore, CRI may be germline encoded, but the product of more than one $V_H$ or $V_L$ germline gene and therefore, may be markers defining sub-subgroups of a family of genes (Bona et al, 1987, Zanetti et al, 1986 and Bonilla et al, 1990).

An interpretation may be, that, the genes may be selected for expression and so not expressed in a random fashion and therefore may be expressed at a higher frequency than other genes.

**c) Is there a relationship between Vκ/VH association and auto/polyreactivity ?**

*i.* In the murine system, anti-DNA antibodies have been described both utilizing different variable region antibody genes (Trepiccio et al, 1987) and with a biased usage of VH genes (Raddic et al, 1991).

On the other hand, RF in the mouse, have been shown to be generated by a limited set of light chains in association with a diverse group of heavy chains (Schlomchik et al, 1987).

Similarily, in the human system, at least for paraproteins, many VH chains may be used for RF autoantibody synthesis. This is consistent with observations concerning the heterogeneity of human RF heavy chains (Chen et al, 1985). In fact, further observations have revealed that at least two separate Vκ light chains can be used for RF synthesis in humans (Crowley et al, 1988)

An elegant experiment demonstrated the significance of the heavy chain in RF specificity and expression of CRI. It was shown that an antibody bearing a VκIIIb light chain, common to the Wa positive RF, had no RF activity alone, and did not express the Wa id. However, recombination of the heavy chain of a Wa positive IgM RF with the light chain of this antibody, resulted in the introduction of RF activity (Newkirk and Capra, 1989).

Therefore, it would appear that, the expression of Vκ325 light chain does not necessarily have to be associated, or be sufficient, for RF specificity, although RF specificity may be very highly associated with the expression of these light chain CRI in some autoimmune or B cell malignancies (Kipps et al, 1990).

Furthermore, Vκ325 light chains have been detected in antibodies with other specificities (Pons-Estel et al, 1984, Goni et al, 1987, Agnello et al, 1987 and Chen et al, 1986).

*ii.* The IgM Fab site which binds SpA, is found in this study and in others (Young et al, 1984, Jirik et al, 1986 and Biguzzi et al, 1982), on antibodies with diverse specificities and is represented on different light chains.

The SpA specificity may have been evolutionarily conserved in the VHIII gene family to accommodate a strong antibody response against it or a similar organism.

Many microrganisms are encountered at mucosal surfaces and as one might predict, B cells bearing the major RF associated CRI are numerous in tonsil and salivary glands (Fox et al, 1986). RF precursor B cells, in fact, were observed to migrate from the bone marrow to gut-associated lymphoid tissue (GALT) in a 129/SV mouse colony that had been infected with an intestinal parasite (Van Snick et al, 1979).

**d) Evidence for a selective expansion of CD5 B cells**

*i.* Of particular interest in my studies, was the finding of significantly higher frequency of kappa light chain clones belonging to the CD5+ B cell clones expressed the 17.109 CRI, thereby utilizing the Vκ325 germline gene (Radouxet al, 1986). Other studies of light chain subgroup expression, have demonstrated, a relatively high frequency of VκIII subgroup expression in CD5 B-CLL cells (Kipps et al, 1987).

Selective gene rearrangement of such light chain genes and their combining with particular heavy chains, could result in the coexpression of two idiotypes in such a way that

may indicate a particular antibody specificity. This in turn, by id-α-id interaction, would drive the expression of these cells. Such continual cycling of such cells would surely increase their chances of undergoing malignant transformation and could account for the clonal expansion of CD5 B cells in disease such as CLL.

In this light, recent data demonstrated, by comparison with the Ig expressed by non-malignant G6 CRI+ B cells, an apparent restriction in the CDR3 of IgH, expressed by G6 CRI+ CLL. Evidence has been found of constraints in the junctional diversity of the IgH chains expressed in CLL (Duffy et al, submitted, 1992). Nucleotide sequence analysis of G6 CRI+ CLL cells that express the 51P1 germline gene, reveals an unusually long CDR3, and favours the use of JH3 (Yamada et al, 1991). Such IgV gene rearrangement, coupled with the observed potential bias in antibody light and heavy chain pairing, expressed in CLL, ie, the high frequency of expression of G6 and 17.109 (Kipps et al, 1990), may have an influence on the antibody repertoire expressed.

However, my studies have indicated that auto or polyreactivity, although, would appear to be related to the utilization of germline genes, does not appear to be associated with any distinct gene families.

Rather, it appears that these types of specificities are genetically programmed and intrinsic to B cells.

*ii.* A higher than expected frequency of VHIV gene expression was demonstrated in both CD5+ and CD5- cells.

This may reflect an evolutionary selective pressure for VHIV encoded polyreactive antibodies expressing perhaps

resulting in significant implications, for example, sensitivity to clonal deletion or specific T cell subsets.

Alternatively, it may simply be indicative of the genetic elements that mediate preferential utilization, for example, the proximity of the VHIV genes to the DH locus may account for a high frequency of VHIV utilization, early in life (Perlmutter et al, 1985).

Most interesting was my finding of a selective association of a small subset of VHIV genes, characterised by the exclusive expression of the 9G4 CRI (Stevenson et al, 1986) with CD5+ B cells and a preferential expression of the Lc1 CRI (Ono et al, 1987).

Results of sequence analysis of 7 of these clones suggest that all the 9G4 reactive clones have rearranged a single VHIV gene, 4-21 (Deane et al, submitted). Furthermore, preliminary data has confirmed that these B cells have cold agglutinin activity (Stevenson et al, unpublished). Since these B cells do not have the reported associated with VκIIIb, reported for VHIV paraproteins (Capra et al, 1982), the light chain does not appear to be necessary for this particular specificity.

The V4-21 gene (Sanz et al, 1990) is the least homologous VHIV gene to the other members of the VHIV family. This association between 9G4 and V4-21 has also been described by Pascual et al, 1991, Silberstein et al, 1991. The Lc1 reactive lines rearranged the VHIV gene 2-1 and the closely related gene 71-2, suggesting this CRI recognises a subfamily of VHIV genes. Similar findings have been described in a series of Lc1 reactive B cell malignancies (Pratt et al, 1991).

Furthermore, since the reactivity between 9G4 and

Lc1, is mutually exclusive and the V2-1 and V71-2 and V71-4 have distinct CDR1 and CDR2 regions (Deane et al, submitted 1992), the molecular basis for Lc1 reactivity may be determined by the portion of the first FR which is common to these genes and distinct from that of the V4-21 group or the remaining VHIV genes.

It is of interest that two of the CD5+ clones in my studies that are Lc1 positive, and so utilize VHIV family genes, also react with the BEG-2β antibody. The BEG-2β antibody has been shown to identify a protein recently mapped to VHIV and VHVI family genes and sequence analysis of the heavy chain of the BEG-2 molecule has reported that the VH71-2 germline gene is the most closely related to VH BEG-2 (Watts et al, 1991). These clones are highly polyreactive and interestingly, these particular VHIV and VHVI gene families are closely related through the "family specific" framework regions recently described (Hillson et al, 1990) and which comprise part of the VHIII family. These regions are sequences that are both family specific and highly conserved between mouse and man, ie, "super families".

**e) What is the significance of selected, expressed immunoglobulin genes by CD5 B cells ?**

*i.* Murine, CD5, CH lymphomas have shared idiotypes (id) (Pennell et al, 1985). However, subsequent data (Pennell et al, 1988), showed from nucleic acid sequencing of immunoglobulin variable region genes, that the frequent occurrence of CRI was not as important as the fact that CH lymphomas express a restricted set of variable region genes.

Interestingly, in the case of follicular B cell

lymphoma, undergoing somatic hypermutation, a particular variable region determinant was conserved, suggesting that this determinant played a role in tumour growth (Con et al, 1987).

*ii.* If the CD5 molecule is an activation marker of B cells (Werner-Favre et al, 1989), then clearly the expansion of unique subsets of B cells will be influenced by encounter with antigen or influenced in their microenvironment.

To this end, if the differentiation of CD5 in vivo, is associated with the loss of surface CD5, as we have observed in vitro with EBV, then the R2.1A2/9G4 CRI encoding genes may be evolutionary selected substrates for mutation on encounter with antigen.

**f) How might the immunoglobulin diversity of CD5 B cells be determined ?**

The use of transgenic mice, both to express chosen immunoglobulin genes (Grosschedl et al, 1985 and Ruscani and Kohler, 1985) and defined "pseudo-self antigens" (Adams et al, 1987), could be useful in defining the CD5 B cell. Immunoglobulin transgenic mice have relatively intact CD5 B cells even when most of their conventional B cells only express the transgene, therefore, endogenous immunoglobulin rearrangements in CD5 B cells are more resistant to suppression by an immunoglobulin transgene (Stall et al, 1988ii). This may demonstrate that the CD5 B cell population is highly selected where endogenous genes provide an advantage over the transgene.

CD5 may be functional, only when co-expressed with

some other, as yet undefined antigen. It is likely that as more surface markers are identified and more sophisticated cell separation techniques become available, B cell sub-populations will be defined, independent of the so called "primary/conventional" population.

It is interesting to consider the chicken in the context of these proposals. It has an established distinct mechanism for the generation of immunoglobulin diversity (Lassila et al, 1988). Perhaps a parallel can be drawn with CD5 B cells and that a "primitive" and a "conventional" B cell lineage coexists in mammals (Herzenberg et al, 1989).

Coutinho and his colleagues, 1988, proposed a "network" model, hypothesising that, in the sterile fetal environment, stimulation by autoantigens and/or idiotype-anti-idiotype interactions would selectively expand these B cells expressing the autoreactive V genes, to form a functional network. With maturation, this proposed network would diminish in size, but remain and interact with those non-autoreactive cells not in the network (Lundkvist et al, 1989).

Autoreactive V genes have been shown to be inherited by most individuals and preferentially expressed during early development (Holmberg et al, 1987, Bona et al, 1988, Fong et al, 1985, Portnoi et al, 1986, Zouali et al, 1988 Kearney et al, 1986, Souroujon et al, 1988, Chen et al,1988, 1989, Dersimonian et al, 1987, Sanz et al, 1989 and Perlmutter et al, 1987).

## 7.4 DO CD5 B CELLS CONTRIBUTE TO AUTOIMMUNE DISEASE ?

The precise function of CD5 B cells in the pathogenesis of autoimmune disease remains unknown, but is is becoming increasingly possible that these cells are able to play a central role in the dysregulation of the immune system, observed in diseases like RA. This is based on a number of observations :

### a) Multispecific antibodies in autoimmune disease

*i.* It has been suggested, as my results support the view, that, the CD5 B cell lineage is responsible for producing germline encoded autoantibodies of IgM class, of low affinity and high cross-reactivity, whereas the "conventional" B cell is responsible for the production of somatically mutated, antigen driven antibodies, of IgG class and high affinity (Carson et al, 1987).

However, in autoimmune diseases like RA, in some cases, there is a higher than normal proportion of "fetal type" CD5 B cells, as I, and others have observed, remaining in the adult repertoire (Youinou et al, 1987, Plater-Zyberk et al, 1985, 1989). Furthermore, CD5 B cells do exist in RA synovium and synovial CD5 B cells can undergo class switching (Plater-Zyberk et al, 1992).

It could be that autoantibodies, seen in rheumatic diseases have undergone affinity maturation due to constant exposure to autoantigens. This hypothesis is in keeping with a recent report demonstrating in RA, the presence of CD5 B cells producing high affinity monoclonal autoantibodies in addition to the multispecific autoantibodies (Burastero et al, 1988).

Furthermore, reports have shown that CD5 B cells in RA, produce RF which are not cross-reactive (Burastero et al, 1988) and can be of isotypes other than IgM (Casali et al, 1987).

*ii.* It has been proposed (Avrameas et al, 1983) that B cells bearing polyspecific antibody receptors could be induced by interaction with a specific antigen to mutate towards a state of high specificity.

A significant number of hybridomas from immunized animals reacted with the immunizing antigen and other self-antigens (Guilbert et al, 1985). Indeed, monoclonal antibodies reported to be specific to different antigens, were encoded by the same or mutated forms of the germline gene that encoded polyspecific natural antibodies (Baccala et al, 1989).

*iii.* It has been reported that, in both mice and men, autoreactive B cell precursors are often in a proliferative state (Fong et al, 1985 and Portnoi et al, 1986), possibly stimulated by autoantigens.

It was recently shown that CD5 B cells respond better to T cell independent stimuli (Zupo et al, 1991), not only does this fit with the notion that CD5 B cells offer the first line of defence against pathogens (Casali et al, 1989), but if the CD5 B cell is easy to stimulate, this may favour variable region gene mutation. This accompanied by selection, could lead to the expansion of such cells carrying an autoimmune repertoire and may be related to the pathogenesis of diseases.

*iv.* Finally, in patients where CD5 B cells spontaneously

proliferate, IgM RF is produced which is monoreactive and has a high affinity for IgG Fc.

Molecular studies revealed that there was extensive somatic point mutations, clustered in the CDR of the variable regions. The location of these mutations and the high replacement to silent ratio (R/S = 5:1), are consistent with the hypothesis that, similar to conventional CD5 B cells, IgM RF CD5 B cells can undergo an antigen driven process of expansion and selection (Kasaian et al, 1992).

**b) Implications for the expression of CRI in autoimmune disease**

The reason for the absence or low level of expression of RF CRI in the polyclonal RF proteins found in RA (Yang et al, 1991), is unclear.

However, it may be that germline encoded RF sequences, hence expression of CRI, may be characteristic of early disease, but that mutation within these genes may lead to a reduced proportion of RF's expressing a CRI with disease progression. Any subtle mutations or minor amino acid substitutions within the CDR's or FR's, may result in a conformational alteration. This would affect the binding affinity of the anti-idiotypic antibodies.

Furthermore, autoantibody associated $V\kappa$ genes, $V\kappa 325$ and $V\kappa 328$, are highly conserved in humans, whereas some autoantibody associated $V_H$ genes, are polymorphic (hv3005 and hv1051), (Chen et al, 1990, Walter et al, 1988, Humphries et al, 1988, Van Dijk, et al, 1989, Souroujon et al, 1989 and Zouali et al, 1989). Although no polymorphism has been found in autoimmune mouse strains (Kofler et al, 1985),

tiny sequence variations in the coding regions, or amino acid substitutions may contribute. For example, as in the beta chain of HLA and susceptibility to insulin dependent diabetes mellitus (IDDM) (Morel et al, 1988).

## 7.5 CD5 B CELLS AS "CARETAKERS" OF TOLERANCE

### a) B cell compartment

A strong case could be made for the view, that somatic hypermutation in the Ig V genes of B lymphocytes takes place in germinal centres, as part of the antigen driven process which forms memory cells, capable of producing high affinity antibodies (reviewed by Nossal, 1988). It seems that precursors to these memory cells are susceptible to tolerance induction for some days after they are activated by antigen (Klinman et al, 1988), presumably to prevent any dangerous anti-self reactivity emerging.

Perhaps it is the role of CD5 autoreactive B cells to induce clonal anergy/abortion within the B cell compartment, particularly towards important self-cell surface antigens.

Healthy and diseased individuals have B lymphocytes that can be activated to produce autoantibodies, by autoantigens (Logtenberg et al, 1989) so, idiotypic determinents expressed on autoantibodies, secreted by CD5+ cells, could well be the target of regulatory mechanisms involved in the maintenance of tolerance to self.

As such, rather than suppression or deletion of these cells, having them in an active state would be beneficial.

### b) T cell compartment

CD5 B cells, represent the majority of infrequent B

cells in the thymus and may be involved in the generation of tolerant clones (negative selection) in the thymus (Inaba et al, 1991). Recognition of processed autoantigen by T cells, at a particular stage of T cell development, may result in the depletion of T cell directed to self.

## 7.6 PROPOSED MODEL FOR THE DEVELOPMENT AND FUNCTION OF CD5 B CELLS.

The model, illustrated in Fig.7.1, proposes that :

**a)** Stem cells (SC) in the fetus, give rise to a predominant population of CD5+ B cells that may populate unique, as yet unidentified, locations in the body. Any B cells which are generated and give rise to high affinity anti-self antibodies, die by apoptosis. Those cells that have a low affinity for self, escape death, possibly "rescued" through the expression of a protein such as bcl-2 (Liu et al, 1991).

**b)** At birth, CD5 B cells will still be the predominant, if not, the only, B cell population, and will be maintained in an activated state by the effects of interleukins (IL2, IL5, IL10?) and the effects of self-stimulation. These activated CD5 B cells, express their sIg and also have the capacity to react with ubiquitous microrganisms (and possibly maternal Ig, transferred across the placenta). As already discussed, these B cells, may enhance an immune response and engage IgG, already bound to foreign antigen and therefore, increase the size of the preformed immune complex.

CD5 B cells can produce low affinity, broad specificity antibody, and may be regulated through a "network" of idiotypic/anti-idiotypic interactions. One could predict, that such constant stimulation, may increase the chance of some cells mutating, and possibly even switching, from low affinity IgM antibodies, to a "higher" affinity IgM, or even IgG. Such events may lead to :

i. the loss of polyreactivity, as the cells may move towards being a more "mature" type cell

ii. the down-regulation of surface CD5 expression, perhaps

through the effects of interleukins secreted (eg. IL4, Freedman et al, 1989 and Werner-Favre et al, 1989). The result of this, may be reflected by the presence of a sub-population of B cells with a unique phenotype, such as the described CD5- CD45RA+ B cells (Kasaian et al, 1992), the human homologue of the Ly1 (B1b) "sister" B cell population (Herzenberg et al, 1989).

c) Lanzavecchia recently showed that B cells with RF activity can effectively process antigen complexed to IgG antibodies. It may be the role of CD5+ RF precursor cells (17.109+ and known to exist in the mantle zone of germinal centres, Kipps et al, 1991), to process and present such complexes. Regulatory mechanisms to control any proliferation of autoreactive cells, would likely involve T cells, or perhaps inhibition through the B cell Fc receptor (Cambier et al, 1987 ).

Furthermore, should the differentiation of CD5 B cells, *in vivo,* be associated with the loss of surface CD5, then it may be that the genes they encode are selected during evolution as substrates for mutation on encounter with antigen. Indeed CD5- B cell clones, expressing diverse VH genes, may be evidence of this (Roudier et al, 1990).

d) Therefore, those cells expressing particular VH genes, on encounter with antigen, will initiate primary follicle or germinal centre (GC) formation and somatic mutation. Again, a clonal expansion of autoreactive cells would be regulated, but a few antibody forming cells may emerge from the germinal centres and eventually become high affinity IgG memory cells that have mutated away from the germline and no longer express their CRI.

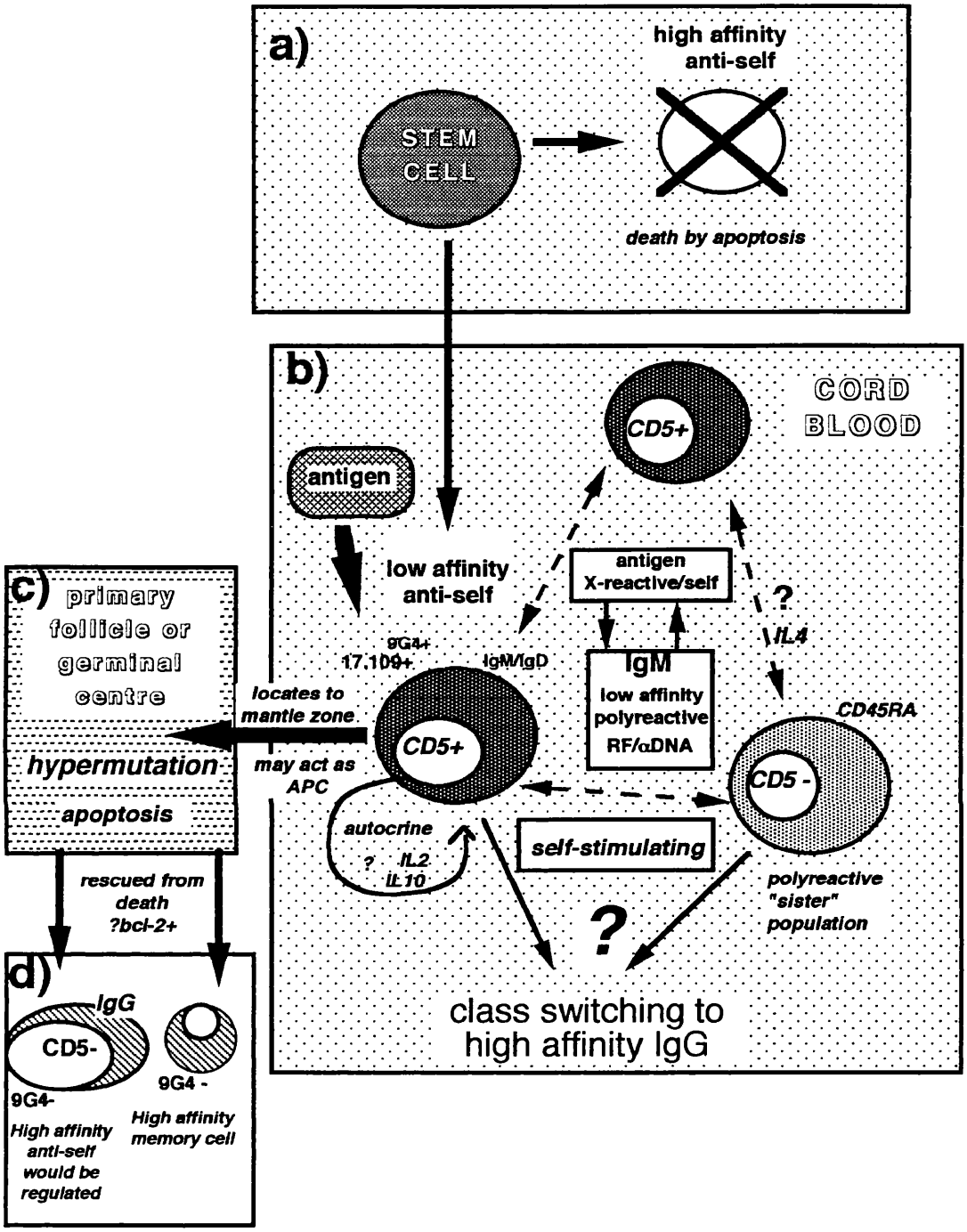


**FIGURE 7.1 PROPOSED MODEL FOR THE DEVELOPMENT AND FUNCTION OF CD5 B CELLS. a)** Stem cells give rise to **b)** CD5 B cells capable of generating low affinity polyreactive anyobody to self or a cross-reactive (X) antigen. These cells are maintained through self-stimulation and autocrine growth factors, through which, they may lose their surface CD5 molecule. **c)** Some express particular CRI (for eg. 9G4 ) and may be stimulated on encounter with antigen to undergo somatic mutation. **d)** This may result in the generation of a high affinity antibody, that no longer expresses the CRI. Some 17.109+ cells may function as antigen presenting cells (APC) and locate in the germinal centres.

## 7.6 FUTURE EXPERIMENTS

Based on the model, outlined above, there are a number of potentially promising areas for future investigation :

1. To test the hypothesis, that the cord blood B cell repertoire, may be fundamentally different to the adult, it will be interesting to compare the VH and VL gene family usage of adult CD5 B cells, with the cord blood data, in these studies to date.

Parallel experiments will analyse native cord blood, before and after transformation by EBV. This will eliminate, as already discussed, any suggestion that the virus is transforming a selected sub-population of cord blood B cells.

Both these studies may be achieved by analysis using dual immunofluorescence and flow cytometry.

2. An interesting question arises concerning the "class switching" potential of these polyreactive natural antibodies, and indeed the nature of the antibodies they would secrete.

It may be possible, using a cocktail of interleukins, to attempt to "class switch" an IgM cord blood clone and examine its specificity and expression of germline genes.

3. Sequence analysis of the cord blood clones will signify if there are any distinctions between the antibody variable region gene repertoire expressed by CD5+ and CD5- cord blood clones, ie, are they the same or could the CD5- B cells be derived from the CD5+ population?

Such a study is currently underway in collaboration with Drs M. Deane and R.A. Mageed, using a number of the CD5+

clones which demonstrated a selective expression of the VHIV associated CRI, are being examined (as discussed in section 7.3c).

4. The molecular basis for polyreactivity still remains unanswered. This will be determined by comparing the sequences of polyreactive antibodies with monoreactive, from cord blood clones.

5. It also, remains to be resolved in the human, if B cells making polyreactive antibodies (CD5 B cells) represent the suggested "primitive" interconnecting set of germline encoded B cells, that may be involved in setting up an idiotypic network early in life (Holmberg et al, 1987). Future studies of connectivity of CD5 B cells will be important.

6. To test the hypothesis, that, CD5 B cells may be involved in initiating autoimmune responses, ie, polyreactive antibodies, may be able to present self-antigen, in an autoimmune state.

B cells with low avidity antigen receptors will be expected to process and present non-self / self antigen to T cells.

7. With regard to the evolution of germline genes, I would also like to test the hypothesis that, the VH families, utilised in early life, represent targets for "superantigens". These antigens would stimulate large numbers of such B cells and may be of microbial origin, as has been described for T cells (Marrack and Kappler, 1990) and Herman et al, 1991).

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# Specificity and Idiotope Expression of IgM Produced by CD5$^+$ and CD5$^-$ Cord Blood B-Cell Clones[a]

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## INTRODUCTION

A normal murine B-cell subpopulation expressing the 67 kDa T-cell marker, Ly-1 (CD5), was first described almost a decade ago.[1] Since then much interest has been generated in this population, as it was shown to spontaneously produce IgM[2] and was responsible for autoantibody production[3] in certain murine models of autoimmune diseases. Furthermore, B-cell tumors were frequently found expressing CD5.[4,5] Similar observations were made in humans where adult CD5$^+$ B cells were shown to be responsible for the production of anti-single-stranded DNA (ssDNA) and anti-IgG-Fc (rheumatoid factor, RF).[6,7] This population has subsequently been shown to produce low avidity polyreactive antibodies,[8] which form the large pool of natural autoantibodies present in normal human sera.[9] Malignant chronic lymphocytic leukemia (CLL) B cells also carry CD5 and show high auto- and polyreactivity revealed by *in vitro* activation with phorbol ester.[10,11] Furthermore, it has been suggested that CD5$^+$ B cells might also be involved in idiotypic interactions as part of the general idiotypic network.[12]

In order to characterize this population more fully, our approach has been to immortalize cord-blood B cells, a rich source of CD5$^+$ B cells,[13,14] with Epstein-Barr virus (EBV) following sorting of cells stained for CD5 and separated from negative cells by flow cytometry.[15–17] EBV-immortalized cell lines were analyzed for $\kappa$ or $\lambda$ light

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TABLE 1. Summary of Reagents Used to Define Vκ and $V_H$ immunoglobulin Subgroups and CRI[a]

| Mouse monoclonal antibody | Association | Paraprotein | CRI family |
|---|---|---|---|
| G6[33] | $V_H$I | Ko | Wa |
| G8[33] | | Ko | Wa |
| D12[33] | $V_H$III | He | He |
| B6[33] | | He | He |
| Lc1[28] | $V_H$IV | — | — |
| R2.1A2[30] | | KAR | — |
| C7/C6[33] | Vκ III/IIIb | Ko | — |
| 17.109[33] | Vκ IIIb | Sie | Wa |

[a] For more details on preparation of reagents and source of paraproteins see refs. 28, 30, and 33.

chains, and antigen specificities were determined by ELISA and immunocytochemistry using a range of autoantigens including IgG-Fc (RF specificity), ssDNA, cardiolipin, histones 1-4, and cytoskeletal components. These methods have previously been described in detail elsewhere.[13,15,17]

A number of studies in mice have suggested that $CD5^+$ B cells might differ in expression of variable region genes from their conventional counterparts.[18–20] Although the definitive answer must come from gene sequences, our initial approach has been to analyze the reactivity of the IgM products of the $CD5^+$ and $CD5^-$ lines with monoclonal antibodies raised against cross-reactive idiotopes (CRI) carried by IgM paraproteins with RF activity, essential mixed cryoglobulinemias, and cold agglutinins. It has been suggested from a number of studies that CRI are the products of germ-line or minimally mutated germ-line gene products.[21,22]

The preparation of monoclonal antibodies (mAb) to various paraproteins and their fine specificities have previously been described in detail,[23,24] and a summary of the reagents used in this study is shown in TABLE 1. Briefly, mAb G6 and G8 recognize CRI associated with the heavy chain of immunoglobulins from the $V_H$ I family.[24,25] Monoclonal antibodies B6 and D12 react with CRI associated with the heavy chains of immunoglobulins from the $V_H$ III family.[26,27] Lc1[28] reacts with the $V_H$ of proteins from the relatively small $V_H$ IV family ($\leq 10$ genes),[29] whereas R2.1A2 was raised against a cold agglutinin paraprotein[30] from the same $V_H$ family.[29] Monoclonal antibody 17-109[31] recognizes a CRI on Vκ IIIb light chains encoded by the Vκ III germ-line gene Kv 325[32] and was kindly provided by D. Carson (Scripps Institute, La Jolla, USA). Monoclonal antibodies C7 and C6 with specificity for Vκ III-[23] and Vκ IIIb-associated isotypic epitopes, respectively,[27] were also used to characterize IgM produced by the B-cell lines.[33] The reactivity of the various mAb was evaluated by ELISA as previously described.[16,33]

IgM from the $CD5^+$ and $CD5^-$ clones was also tested by ELISA and hemagglutination assay for binding to staphylococcal protein A (SpA) to distinguish $V_H$ III family products from other $V_H$ families.[34]

In this paper we refer to the EBV-driven B-cell lines as clones. Whereas we cannot be certain that all of the individual "lines" are monoclonal, a number of pieces of data point toward this. First, the proliferating cells were obtained by culture under limiting

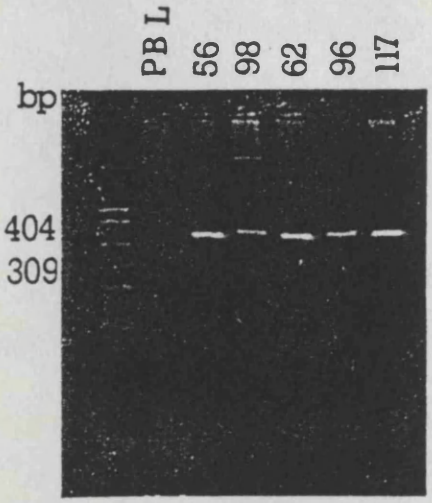


**FIGURE 1.** Agarose gel analysis of $V_H$ family-specific IgH gene rearrangements detected by polymerase chain reaction (PCR). DNA from normal blood lymphocytes (PBL) and from cord-blood clones 56, 98, 62, and 117 was amplified under conditions adapted for each family-specific primer set in order to eliminate nonspecific amplification products.

dilution conditions; second, the lines were shown to express only one light chain type; third, they reacted with monoclonal antiidiotopes associated with only one $V_H$ family (as described in this study); and fourth, in preliminary experiments using genomic DNA from 7 of the $V_H$ IV family-expressing clones as determined by mAb, single rearrangement of DNA from only this $V_H$ family was detected using PCR analysis (FIG. 1).

This paper reviews our studies on the specificity and idiotope usage of $CD5^+$ and $CD5^-$ clones, some of which have been published in more detail elsewhere.[15–17]

## SPECIFICITIES OF $CD5^+$ AND $CD5^-$ CLONES

### *Both $CD5^-$ and $CD5^-$ Clones Can Secrete Auto- and Polyreactive Antibodies*

A high frequency of clones derived from both $CD5^+$ and $CD5^-$ cord-blood B cells reacted with the autoantigen panel tested. Examples of some of the specificities of representative clones are shown in FIGURE 2. A more detailed description of reactivities of the $CD5^+$ and $CD5^-$ clones to a panel of autoantigens has been reported elsewhere.[15] IgM from both $\kappa$ and $\lambda$ light chain clones reacted with IgG, ssDNA, or type I/II collagen. Many clones also produced polyreactive IgM as defined by their reactivity with two or more unrelated autoantigens (*e.g.* ssDNA and IgG-Fc), whereas occasionally clones were found that failed to react with the autoantigen panel tested. In defining specificities for IgG-Fc, there were significantly more $CD5^+$ clones that reacted with IgG-Fc compared with CD5-clones (43/86 $CD5^+$) and 21/75 $CD5^-$ clones; $p < 0.01$). Similarly, 44 of 86 $CD5^+$ and 18 of 75 $CD5^-$ clones produced anti-ssDNA, ($p < 0.001$). Furthermore, clones with ssDNA specificity ($p < 0.01$) and RF activity, together with ssDNA specificity ($p < 0.02$), were significantly more frequent in the $\kappa$ than $\lambda$ clones in the $CD5^+$ population. In this same study it was shown that IgM from at least six $CD5^+$ and $CD5^-$ clones was of low avidity.[15] Such characteristics have been attributed to antibodies derived from unimmunized individuals or mice and have been designated "natural autoantibodies."[9,35]

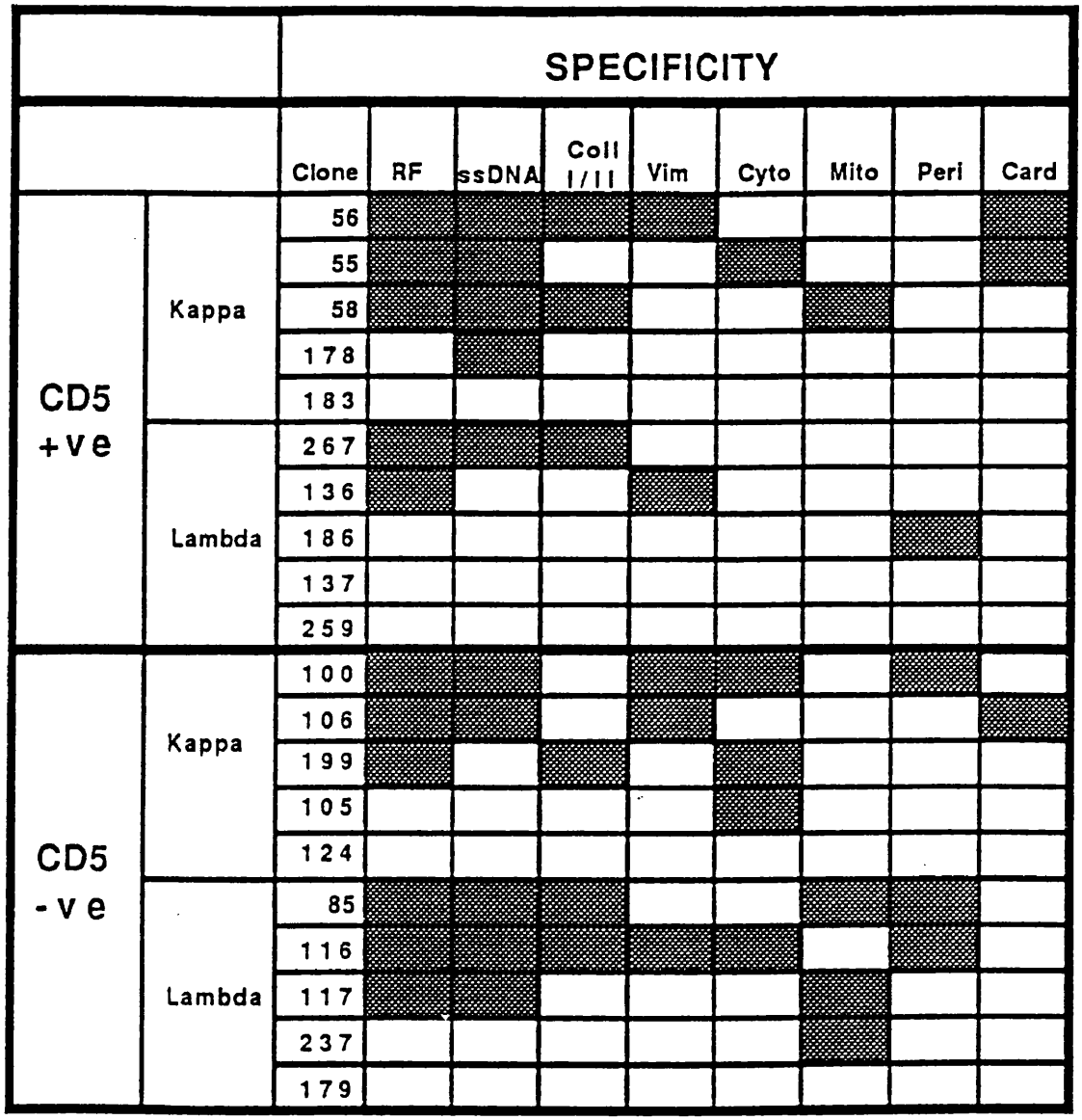


**FIGURE 2.** Specificities of some $CD5^+$ and $CD5^-$ clones. Autoantigen specificities were determined by ELISA and cytochemical staining previously described in detail.[13,15] The majority of clones derived from the $CD5^+$ and $CD5^-$ cord-blood B cells were specific for single autoantigens or were polyreactive. A small number failed to bind to any autoantigen in the panel. RF, rheumatoid factor; ssDNA, single-stranded DNA; Coll I/II, collagen type I and II; Vim, vimentin; Cyto, cytokeratin; Mito, mitochondrion; Peri, perinuclear; Card, cardiolipin.

## EXPRESSION OF $V\kappa$ AND $V_H$ FAMILY-ASSOCIATED IDIOTOPES BY $CD5^+$ AND $CD5^-$ CLONES

### *More Frequent Expression of 17-109 by $CD5^+$ B-Cell Clones than by $CD5^-$ B-Cell Clones*

Of 86 clones derived from $CD5^+$ B cells, 54 used $\kappa$ chains (63%) while significantly fewer, 30 of 75 (40%), $CD5^-$ B-cell clones used this light chain isotype ($p < 0.01$: data not shown). The $\kappa : \lambda$ ratio of the $CD5^+$ clones was higher than that seen with the $CD5^-$ clones.

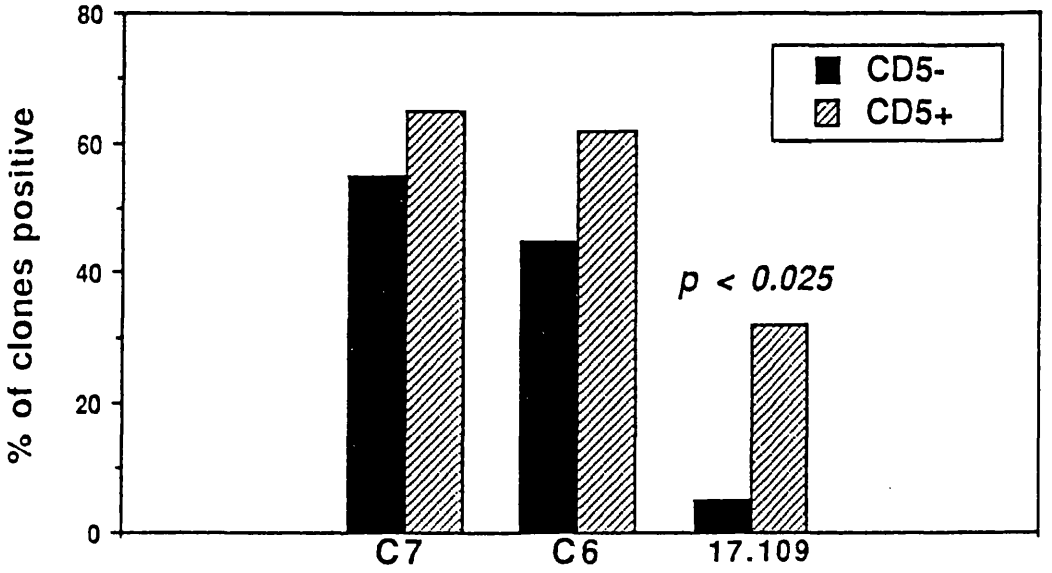


FIGURE 3. Vκ III subgroup and Vκ IIIb sub-subgroup analysis of IgM produced by $CD5^+$ and $CD5^-$ κ clones. Monoclonal antibodies to Vκ III subgroup-associated isotypic determinants (C7); Vκ IIIb sub-subgroup (C6) and Vκ IIIb sub-subgroup-associated CRI 17-109 were used in ELISA. IgM from 20 $CD5^-$ and 34 $CD5^+$ B-cell clones was examined. Significantly more of the $CD5^+$ clones produced IgM-expressing 17-109.

A high percentage, 65% of $CD5^+$ and 55% of $CD5^-$ κ clones, were of the Vκ III subgroup (FIG. 3). In both groups, > 75% were of the Vκ IIIb sub-subgroup as detected using the monoclonal antibody C6. A significantly higher frequency of the $CD5^+$ clones (11 of 34), however, expressed 17-109 CRI ($p < 0.025$) than the $CD5^-$ clones (1 of 20).

Of the $17\text{-}109^-$ κ clones analyzed, none were combined with the $V_H$I-associated idiotopes, 5/11 were associated with SpA-binding, but none were associated with the $V_H$ III family idiotopes B6 and D12. Of the rest, 3 were associated with $V_H$ IV-associated idiotopes. Normal B cells expressing 17-109 have been identified in the fetal spleen and are present at high frequency early in ontogeny.[36] These $17\text{-}109^+$ cells also appeared to express the $V_H$I-associated idiotope G6, although no clones with this phenotype were identified in our study. The idiotope recognized by the 17-109 antibody is coded for by the Kv 325 gene or a minimally mutated form of this gene,[32] and has been shown to be expressed in leukemic B cells from many patients with chronic lymphocytic leukemia.[37] Many of these malignant tumors have been shown to produce auto- and polyreactive antibodies.[10,11]

### *Similar Frequencies of Expression of $V_H$ I-Associated Idiotope G8 and the $V_H$ III Gene Family-Associated Idiotopes B6 and D12 by $CD5^+$ and $CD5^-$ Clones*

IgM from none of the 40 $CD5^+$ and 45 $CD5^-$ clones examined expressed the $V_H$ I-associated idiotope G6 (data not shown). IgM from 2 $CD5^+$ and 2 $CD5^-$ κ clones,

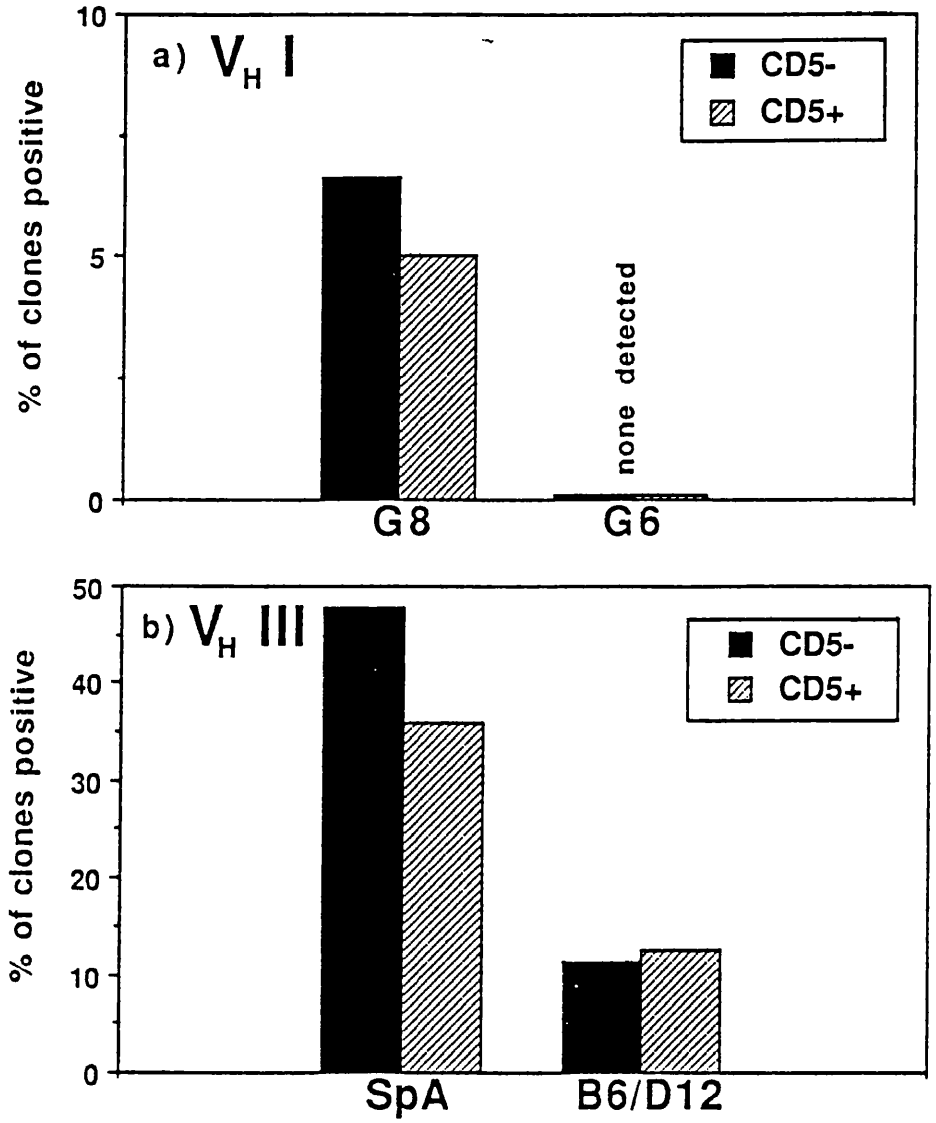


**FIGURE 4a and b.** Equal representation of $V_H$ I- and $V_H$ III-associated CRI by $CD5^+$ and $CD5^-$ clones. Monoclonal antibody G8 ($V_H$ I-associated idiotope), and B6 and D12 ($V_H$ III-associated idiotopes) were used in the ELISA. IgM from 45 $CD5^-$ and 40 $CD5^+$ clones was examined. IgM from 46 $CD5^-$ and 47 $CD5^+$ B-cell clones was analyzed for binding to SpA to determine their $V_H$ III-associated expression.

however, expressed the G8 idiotope (FIG. 4a). All 4 of these $G8^+$ clones were associated with κ chains using VκIII family genes, and 2 were VκIIIb-positive (data not shown). One λ clone expressing G8 was detected in the $CD5^-$ group.

IgM from a relatively large number of the $CD5^+$ and $CD5^-$ clones bound SpA (FIG. 4b). There was no significant difference (at the 5% level) in the frequency of SpA binding between $CD5^+$ (16/47) and $CD5^-$ clones (22/46), but there was a trend towards a decreased frequency of SpA binding by IgM produced by $CD5^+$ clones, perhaps reflecting the relative use of other $V_H$ families by this population. Both κ and λ light chain clones were equally represented in the $SpA^+$ groups, and no differences were seen in frequencies of $SpA^+$ clones expressing κ light subgroups in $CD5^+$ compared with $CD5^-$ clones.

The $V_H$ III-associated idiotopes recognized by mAb B6 and D12 were coexpressed by the same clones in all cases except 3 λ $CD5^-$ clones that were B6 negative but D12 positive. We have previously found λ cord-blood clones and in particular λ fetal-liver

clones positive for D12 but not B6.[33] In this paper we hypothesized that because the idiotope recognized by D12 is so widely used, it might mean that the genes encoding it are of major functional importance. The $V_H$ III family is the largest $V_H$ family in humans and probably comprises many gene subfamilies.[38] Our finding of idiotopes recognized by mAb B6 and D12 on $CD5^+$ and $CD5^-$ cord-blood clones is consistent with their being encoded by germ-line genes. In a previous study we also showed that a $V_H$ III-associated idiotype 16/6,[39] encoded by the $V_H$ 26 germ-line gene,[40] was expressed by 4% of cord-blood derived EBV B-cell lines.[41] This latter idiotype was previously associated with anti-DNA activity, while mAb B6 and D12 were originally prepared against a RF paraprotein from the $V_H$ III family. Although many of the $B6/D12^+$ clones from both $CD5^+$ and $CD5^-$ clones have RF activity, some clearly do not (TABLE 2). Thus these idiotopes, per se, are not sufficient to confer RF activity on IgM antibodies of the $V_H$ III family.

### *Expression of $V_H$ IV-Associated Idiotopes Recognized by R2.1A2 but Not Lc1 Is Restricted to IgM from the $CD5^+$ Clones*

Twelve of 47 (26%) $CD5^-$ clones express the idiotope recognized by Lc1, whereas 7/46 (15%) $CD5^+$ clones were positive for this idiotope. Only IgM from the $CD5^+$ clones (11/46: 24%), however, expressed the idiotope recognized by mAb R2.1A2 (FIG. 5. $p < 0.01$ compared with the $CD5^-$ group). Identical results were obtained for the idiotope recognized by mAb 9G4 raised in rats against a cold agglutinin paraprotein[42] (data not shown). Within the $CD5^+$ population, a higher, but not significant, proportion of $\kappa$ (14/30) than $\lambda$ clones (4/16) were positive for $V_H$ IV-associated idiotopes recognized by both Lc1 or R2.1A2 monoclonal antibodies.

Because the $V_H$ IV family probably contains a small number of genes ($\leq 10$) with a high degree of internal homology (at least 91.5% between the most distant members to a consensus $V_H$ IV sequence), it is likely that the germ-line repertoire would encode relatively few CRI. Monoclonal antibodies R2.1A2 and 9G4 both recognize CRI on IgM from the same clone,[16] while Lc1 reacts with a mutually exclusive CRI on IgM from other clones.[16] Although these CRI cannot be formally assigned to a particular $V_H$ family gene from the $V_H$ IV family, recent data have indicated an association of these CRI with different germ-line genes.[43,44] On the one hand, CRI recognized by Lc1 may be a phenotypic marker of 71.2 and 71.4 and related genes, while R2.1A2/9G4 may be markers for the 4-21 and related genes. These two major sets of germ-line genes might represent two different subfamilies within the $V_H$ IV family.[16] It is of interest that two of the $V_H$ IV-positive clones described in this study react with BEG2 antibody (MacKenzie, Isenberg, and Lydyard, unpublished observations), which is another idiotype recognized by BEG2 antibodies prepared against IgM from an anti-ssDNA fetal liver clone.[45] It has recently been mapped to $V_H$ IV and $V_H$ VI family genes.[46]

The frequency of clones described here using genes from the relatively small $V_H$ IV family is consistent with the data of Sanz *et al.*[44] who showed a high frequency of $V_H$ IV usage in EBV-immortalized adult $CD5^+$ B-cell clones. Furthermore, this is not dissimilar to the frequency of $V_H$ IV-family usage in fetal B cells estimated to be about 21 percent.[47] In addition to the high frequency of $V_H$ IV-family genes used by the total clones, the $CD5^+$ clones had a higher (but not significant) frequency of these family genes than their $CD5^-$ counterparts. From the data on $R2.1A2^+$ clones, it would appear that the $V_H$ region of IgM from $CD5^+$ clones is encoded by a single gene or small number of genes within the $V_H$ IV family for which a CRI, associated with anti-I-carbohydrate antigen specificity, is a phenotypic marker. Interestingly, although 8 of 11 $R2.1A2^+$ clones were associated with $\kappa$ light chains, 7 of these were not of the $V\kappa$

TABLE 2. Autoreactivities of $CD5^+$ and $CD5^-$ Cord-Blood Clones Using Different $V_H$ Family-Associated Idiotopes[a]

| | $CD5^+$ | | | | | $CD5^-$ | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $V_HI$ | $V_HIII$ | | $V_HIV$ | | $V_HI$ | $V_HIII$ | | $V_HIV$ | |
| Specificity | G8 | SpA | B6/D12 | Lc1 | R2.1A2 | G8 | SpA | B6/D12 | Lc1 | R2.1A2 |
| Fc | 1/2 | 8/15 | 3/4 | 3/7 | 10/11 | 2/3 | 6/21 | 3/5 | 5/12 | 0 |
| ssDNA | 1/2 | 6/15 | 3/4 | 5/7 | 10/11 | 2/3 | 4/21 | 1/5 | 5/12 | 0 |
| polyreactive ≥2 antigens | 1/2 | 6/15 | 3/4 | 3/7 | 10/11 | 3/3 | 8/21 | 4/5 | 6/12 | 0 |

[a] Summary of specificities of antibodies from the different clones for Fc of human IgG, ssDNA, and two or more autoantigens (polyreactive). Note that clones using any of the $V_H$ family genes reacted with these autoantigens and were polyreactive.

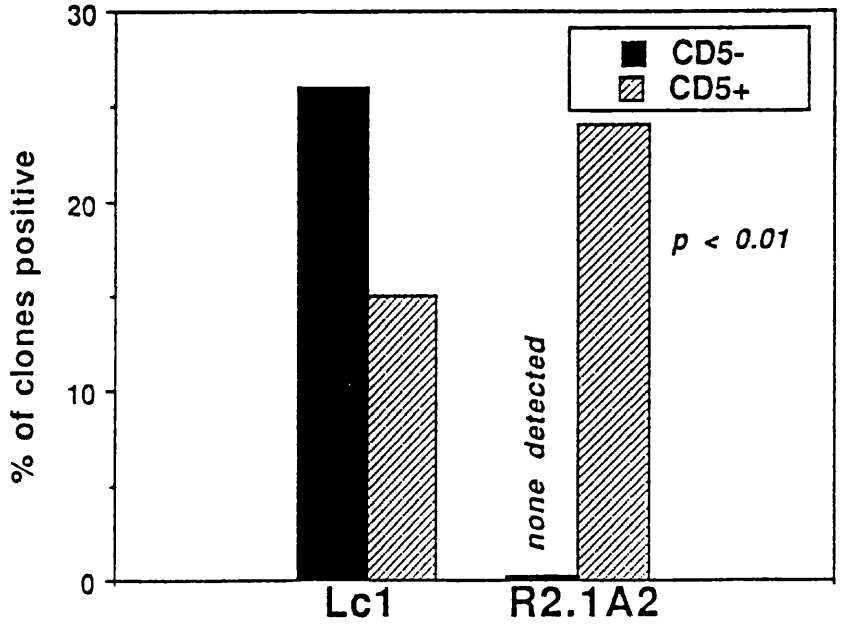


FIGURE 5. Frequency of $CD5^-$ and $CD5^-$ clones expressing $V_H$IV-associated idiotopes. Monoclonals Lc1 and R2.1A2 were used to identify antibodies from 47 $CD5^-$ and 46 $CD5^+$ B-cell clones. Note that only $CD5^+$ clones were identified expressing idiotopes recognized by R2.1A2.

III subgroup (data not shown), which is contrary to the reported association for $V_H$ IV heavy chains and Vκ III light chains in cold agglutinins.[48] One clone was of the Vκ IIIb sub-subgroup, which expressed the idiotope recognized by mAb 17-109.

Over half of the $Lc1^+$ clones in both the $CD5^+$ groups and close to half in the $CD5^-$ group had RF activity (TABLE 2). Some were also polyreactive. In the $CD5^+$ group, however, the $R2.1A2^-$ clones were virtually all polyreactive (10/11). That these were different clones and not simply derived from the same clone is unlikely because there are differences in the $V_H$ and D gene sequences in at least four clones so far studied (Deane *et al.*, unpublished observations).

In conclusion, our data indicate that a higher frequency of autoreactive and polyreactive B cells exist within the $CD5^+$ compared with the $CD5^-$ population. This auto- and polyreactivity does not appear to be the property of any one $V_H$ family. In this regard, murine DNA autoantibodies have been described that use different $V_H$ genes,[49] although there might be a potentially biased usage of $V_H$ genes. The light chain, however, contributes to the binding of the anti-DNA antibodies.[50] Auto- and polyreactivity also occurs with different $V_H$ families with either κ or λ chains (TABLE 2). This non-$V_H$ family-associated polyreactivity might be related to charge effects due to possession of certain polar amino acids, as postulated by Avrameas.[51] This will only be clarified by detailed sequence data. Of particular interest is the finding in this study of the increase in frequency of 17-109 expression in $CD5^+$ clones and the restricted $V_H$ IV subfamily usage. The finding of specific $V_H$ gene usage by the $CD5^+$ population might be interpreted as evidence for a specific subset that becomes selected during development. The driving force for this is unknown but could be exogenous or more likely endogenous self-antigens, including possible complementary idiotopes carried by other lymphocytes.[52] In this light, a high frequency of cord-blood B cells has been shown to express the B-cell activation marker CD78 (F. Yüksel and P.M. Lydyard *et al.*, unpublished observations). The data on cord-blood $CD5^+$ and $CD5^-$ B cells reported here appears in contrast to those of adult $CD5^+$ B cells in which autoreactivity

and polyreactivity were somewhat associated with the $CD5^+$ population.[8,17,18] Our interpretation of this is that the adult B-cell repertoire contains many $CD5^-$ B cells with high-affinity surface Ig receptors resulting from continuous exposure to environmental antigens. The adult $CD5^+$ population, however, is equivalent to the majority of cord-blood B cells that have not been exposed to such environmental antigens. Most of the cord-blood B cells are $CD5^+$, and at least some of those that are $CD5^-$ might be "mutating" away from polyreactivity to more restricted specificity. Sequence analysis of these clones will shed further light on this problem and the mechanism by which polyreactive $CD5^+$ cells might give rise to $CD5^-$ cells.

## SUMMARY

Epstein-Barr virus (EBV)-immortalized monoclonal B-cell lines were established from $CD5^+$ and $CD5^-$ cord-blood B cells. IgM from many of both $CD5^+$ and $CD5^-$ clones reacted with IgG-Fc, ssDNA, and a variety of other autoantigens. More $CD5^+$ B cells that used light chains of the $\kappa$ isotype reacted with IgG-Fc and ssDNA than $\kappa$-bearing $CD5^-$ B cells. Because many of the clones reacted with IgG-Fc, they were analyzed for the expression of cross-reactive idiotypes (CRI) associated with rheumatoid factor and cold agglutinin paraproteins using murine antibodies (mAb) recognizing $V\kappa$ and $V_H$ subgroup-associated determinants.

Expression of the $V\kappa$ IIIb sub-subgroup-associated idiotope recognized by 17.109 mAb was expressed at significantly higher frequency (32%; $p < 0.05$) and IgM antibodies derived from the $CD5^+$ compared with the $CD5^-$ clones (5%). Both $CD5^+$ and $CD5^-$ clones expressed the RF paraprotein-associated idiotope recognized by G8 mAb to the same extent. Similar results were obtained using binding to SpA as a marker of $V_H$ III family usage. Furthermore, no differences in frequency of expression of RF paraprotein-associated idiotopes recognized by B6 and/or D12, and characteristic of some antibodies using $V_H$ III family genes, were found between the $CD5^+$ and $CD5^-$ populations. Although a higher than expected frequency of $V_H$ IV-gene expression was demonstrated (around 30%) in both $CD5^+$ and $CD5^-$ cells, there were differences in expression of CRI recognized by mAb Lc1 and R2.1A2 with specificities for two $V_H$ IV subfamilies. While some $CD5^+$ and $CD5^-$ clones were identified in which their IgM reacted with mAb Lc1, only $CD5^+$ clones were recognized by another mAb R2.1A2.

Analysis of the relationships between antigen specificities and $V\kappa$- and $V_H$-family gene usage indicated that auto- or polyreactivity was not associated with $V\kappa$ III nor any particular $V_H$ family. The higher frequency of the $V\kappa$ IIIb sub-subgroup-associated idiotope recognized by 17-109 in the $CD5^+$ clones and the association of $CD5^+$ B cells with the $V_H$ IV subfamily recognized by mAb R2.1A2 and 9G4 may suggest that $CD5^+$ B cells in cord blood are expanded as a result of recruitment within the fetal environment.

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# Auto- and Polyreactivity of IgM from CD5$^+$ and CD5$^-$ Cord Blood B Cells

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The presence of the CD5 (67 kDa) molecule on the surface of B cells has been considered a marker for cells producing auto- and polyreactive antibodies. Cord blood B lymphocytes (rich in CD5$^+$ B cells) have been sorted into CD5 positive and negative populations by flow cytometry using monoclonal antibodies to CD20 and CD5. Clones of these populations were obtained by immortalization with Epstein–Barr virus. Clones derived from both CD5$^+$ and CD5$^-$ B cells produced IgM which was auto- and polyreactive with a higher frequency of these specificities in the CD5$^+$ population. These data indicate that expression of surface CD5 on cord blood B cells is not a definitive marker of an auto/polyreactive population.

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A subpopulation of B cells expresses a 67 kDa surface molecule (CD5) which is normally found on the majority of T cells [1, 16]. Early data indicated that this B-cell subpopulation is responsible for the spontaneous IgM production of NZB lymphocytes in vitro [12] and is the major producer of autoantibodies to single-stranded DNA (ssDNA) and IgG-Fc (rheumatoid factors; RF) in man [3, 10]. Furthermore, chronic lymphocytic leukaemic B cells expressing CD5 [2] and normal adult CD5$^+$ B cells have been shown to produce mainly polyreactive antibodies [17]. However, the presence of the CD5 molecule as a marker for a subpopulation of B cells has recently been contested following the observations that it can be modulated by activation of human B cells [5, 7, 8, 22].

Our previous studies showed a high proportion of CD5$^+$ B cells in cord blood and that Epstein–Barr virus (EBV) clones derived from this source frequently produced autoreactive and polyreactive antibodies [15]. Some of these polyreactive clones were negative for surface CD5, suggesting that both surface CD5$^+$ and CD5$^-$ cord blood populations might produce polyreactive antibodies. However, further analysis revealed the presence of mRNA for CD5 in some of these CD5 surface negative and polyreactive B-cell clones, which indicated that these clones were of CD5 origin [18].

To clarify the repertoires of cord blood CD5$^+$ and CD5$^-$ B cells we have sorted the cells prior to cloning with EBV and analysed the resulting clones for autoantibody specificity.

Our data indicate that IgM from both CD5$^+$ and CD5$^-$ cord blood B-cell clones can be auto- and polyreactive but that the frequency of clones producing autoantibodies is significantly higher in the CD5$^+$ population.

## MATERIALS AND METHODS

*Preparation of cells.* Umbilical cord blood from three full-term donors were used in this study. Cord blood mononuclear cells were prepared from the blood by centrifugation over Ficoll–Hypaque (F-H) gradients. After treatment with carbonyl-iron to deplete the monocyte population, the mononuclear cells were

further enriched for B cells by sheep rosetting, as previously described [13]. Adult peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers by F-H centrifugation. Washed PBMC cells were irradiated (40 Gy) in a cobalt source and used as feeder cells.

*Preparation of $CD5^+$ and $CD5^-$ B-cell populations.* Enriched cord blood B lymphocytes were reacted with fluorescein-labelled Leu-16 mouse monoclonal antibody to CD20 (Becton-Dickinson, Mountain View, Calif., USA) and phycoerythrin-labelled Leu-1 mouse monoclonal antibody to CD5 (Becton-Dickinson, Mountain View, Calif., USA). After washing the cells were applied to a cell sorter (FACStar, Becton-Dickinson Immunocytometric Systems). Lymphocytes displaying green fluorescence only ($CD20^+$, $CD5^-$ cells) and those displaying both green and red fluorescence ($CD20^+$, $CD5^+$ cells) were 'gated' and sorted as $CD5^-$ and $CD5^+$ B cells, respectively. The degree of discrimination achieved in the sorting procedure was verified by reapplication of the sorted cells to the FACScan analyser (Becton-Dickinson Immunocytometric Systems).

*Epstein–Barr virus infection and production of B-cell clones.* Sorted $CD5^+$ and $CD5^-$ B lymphocytes were cultured in RPMI containing 10% fetal calf serum, supplemented with penicillin and streptomycin (complete medium) at 37°C and 5% $CO_2$. Supernatant from the B95-8 Marmoset line was added at 200 $\mu$l/$10^6$ cells as the source of Epstein–Barr virus (EBV), as previously described [15]. Briefly, EBV-infected cells (0.2–100 cells per well) were seeded into 96-well microplates (Nunc, Roskilde, Denmark) in complete medium together with $2 \times 10^4$ irradiated adult PBMC per well. Plates were incubated for 2–7 weeks at 37°C and 5% $CO_2$ and supernatants from wells containing growing cells assayed for IgM using an ELISA. Plates containing <33% of the wells positive for growth and IgM secretion were selected and further assayed for light chain restriction to determine clonality, as previously described [15].

*Antibody assays*

*IgM assays.* These were performed by ELISA as previously described [15]. All supernatants used for specificity analysis contained > 500 ng/ml IgM. In some experiments the IgM was purified from the supernatants.

*$\kappa$ and $\lambda$ light chain analysis of IgM in supernatants.* 96-well microtitre plates were coated with 100 $\mu$l of 1/1000 dilution of goat anti-human IgM (Tago, Burlingame, CA, USA) in phosphate-buffered saline (PBS) by incubation for 1 h at 37°C and then 4°C overnight. Plates were washed with PBS containing 0.5% Tween (PBS/Tween, Sigma, St. Louis, MO, USA) and blocked with PBS containing 0.5% bovine serum albumin (PBS/BSA) for 1 h at room temperature (RT). After washing with PBS/Tween, 100 $\mu$l of supernatant were added to each well and incubated for 1 h at 37°C and 1 h at 4°C. Wells were then washed with PBS/Tween and 100 $\mu$l added of either 1:1000 goat anti-human kappa or anti-human lambda alkaline phosphatase conjugate (Sigma, St Louis, Mo, U.S.A.) in PBS/Tween containing 1% BSA. The plates were then incubated overnight at room temperature. After washing, the plates were developed with 60 $\mu$l of substrate (p-nitro phenyl phosphate disodium) at 1 mg/ml in ELISA assay mix ($Na_2CO_3$ 0.05M./$MgCl_2.6H_2O$, $10^{-3}$M) for 30 min at RT. The reaction was stopped by the addition of 60 $\mu$l 1N NaOH. The optical density (OD) was read on a microELISA reader at 405 nm (Dynatech, Billinghurst, Sussex, UK).

*Detection of autoantibodies*

*Rheumatoid factor (RF) assay.* This ELISA has previously been described [15]. In all assays, plates were coated with 10 $\mu$g/ml of human IgG1 myeloma (a generous gift from Professor M. W. Fanger and Dr L. Shen, Dartmouth Medical School, Hanover, NH, USA). Supernatants were considered to be positive for rheumatoid factor when the OD values were > mean + 2SD above control values obtained using culture medium alone.

*Collagen type I and II antibody assays.* These were detected by ELISA [15]. Bovine types I and II collagen were a kind gift from Dr K. Morgan, University of Manchester, UK). Supernatants were considered to be positive when the OD values were > mean + 2 SD above control values.

*Thyroglobulin antibody assay.* IgM supernatants were tested for antibodies to thyroglobulin by indirect agglutination using an in vitro diagnostic kit, Serodia-ATG (Fujirebio Inc., Tokyo, Japan).

*Single-stranded DNA (ssDNA) antibody assays.* These were carried out by ELISA as previously reported [15] and supernatants were considered to be positive for anti-ssDNA when the OD values exceeded the mean + 2 SD of the negative controls.

*Anticardiolipin antibodies.* These were also detected by ELISA as previously described [15] and samples with ODs > 2 SD above the mean of background levels (irrelevant proteins or medium alone) were considered positive. Control sera were kindly provided by Dr E. N. Harris (RPMS, London, UK).

*Histone antibody assays.* Antibodies to histones were detected by ELISA using the Nucleoscreen kit (Neosystem, Strasbourg, France) as previously described [15]. Values > 2 SD above the mean of background levels (irrelevant proteins or medium alone) were considered positive. Supernatants are shown as positive for histones if reactivity is shown against one or more individual histone.

*Antibodies to tubulin, actin, vimentin, golgi and cytokeratin.* These were detected in a cell line IMR-33 derived from a gerbil fibrosarcoma (American Type Culture Collection, Rockville, MD, USA), as previously reported [9]. Antibodies to vimentin were confirmed by the perinuclear pattern of staining following colchicine pretreatment.

*Antinuclear (ANA) and nucleolar antibodies.* These were detected by indirect immunoflourescence using HEP-2 cells [9].

*Anti-perinuclear antibodies.* These were detected by immunofluorescence using human buccal cells, as previously described [23].

*Blocking experiments.* IgM was purified using ammonium sulphate precipitation [14] followed by affinity chromatography using an $F(ab)_2$ anti-human IgM (Sigma) column coupled to CNBr-activated Sepharose

4B (Pharmacia, Uppsala, Sweden; 19). Samples of PBS/Tween containing 1% BSA and 2.5 μg of a given monoclonal antibody (MoAb) were mixed with samples of PBS/Tween containing 1% BSA and increasing amounts of soluble IgG. A concentration of 2.5 μg was chosen since this was on the ascending part of the binding curve. After 18 h at room temperature, the various mixtures were transferred into ELISA plates precoated with buffer containing 10 μg/ml IgG and the assay carried out as described already. The binding activity of the MoAb observed in the presence of soluble ligand is expressed as a percentage of the binding activity of the antibody measured in the absence of any soluble ligand (100% binding activity).

*Statistical analysis.* This was carried out by the chi-square test, with Yates' correction when required and Fisher's test for small numbers.

## RESULTS

### *CD5+ and CD5− sorted B cells used for cloning*

B cells expressing the highest density of CD5 were used as the starting population for CD5+ cells. The most negative B cells were chosen for the CD5− starting population. Figure 1 shows the gated populations on the cell sorter of one of the three sorts included in this study.

### *Frequency of κ and λ bearing IgM clones derived from CD5+ and CD5− cord blood B cells*

The light chain restriction of the clones derived from both CD5+ and CD5− cord B cells is shown in Table 1. Higher frequencies of κ clones were noted in the CD5+ population compared with CD5− population (Table 1; $P<0.01$). κ:λ ratios were 1.7:1 and 0.7:1, respectively.

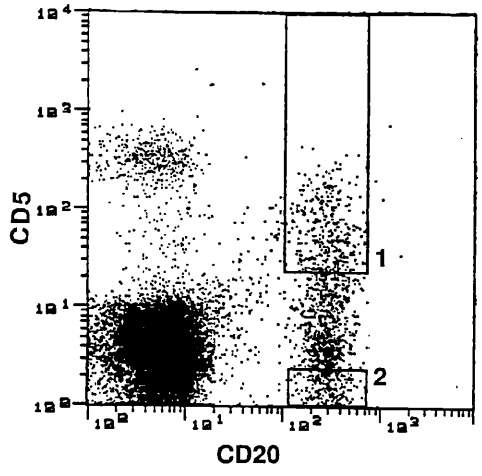



FIG. 1. Gating conditions used to sort cord blood cells. Gate 1, CD5+ B cells; Gate 2, CD5− B cells.

TABLE 1. Light chain restriction of CD5+ and CD5− cord blood clones.

| Clone origin | Total no. | No. (%) κ | No. (%) λ | κ:λ ratio |
|---|---|---|---|---|
| CD5+ | 86 | 54 (63) | 32 (37) | 1.7:1 |
| CD5− | 75 | 30 (40) | 45 (60) | 0.7:1 |

### *Both CD5+ and CD5− clones are autoreactive and polyreactive*

Table 2 shows the relative numbers of clones with specifities for the different autoantigens of the total examined. A high frequency of clones reacted with IgG, ssDNA and type I/II collagen (e.g., 31κ and 12λ out of 86 CD5+ clones), confirming our previous data obtained with unseparated cord blood B-cell populations [15]. Many were also polyreactive, as defined by reactivity with two or more unrelated antigens (e.g. IgG and ssDNA). Both CD5+ and CD5− cord blood B cells reacted with one or more autoantigen, but clones derived from the CD5− B cells had a significantly lower frequency of this activity (Table 2). Forty-three of 86 CD5+ clones but only three of 75 CD5− clones produced RF ($P<0.01$). In addition, 44 of 86 CD5+ and 18 of 75 CD5− clones produced anti-ssDNA ($P<0.001$). Furthermore, ssDNA specificity ($P<0.01$) and RF with ssDNA ($P<0.02$) were significantly more frequent in the κ than λ clones in the CD5+ population.

To emphasize the similarities in specificities of the autoantigen-specific clones within the CD5+ and CD5− populations, individual specificities of

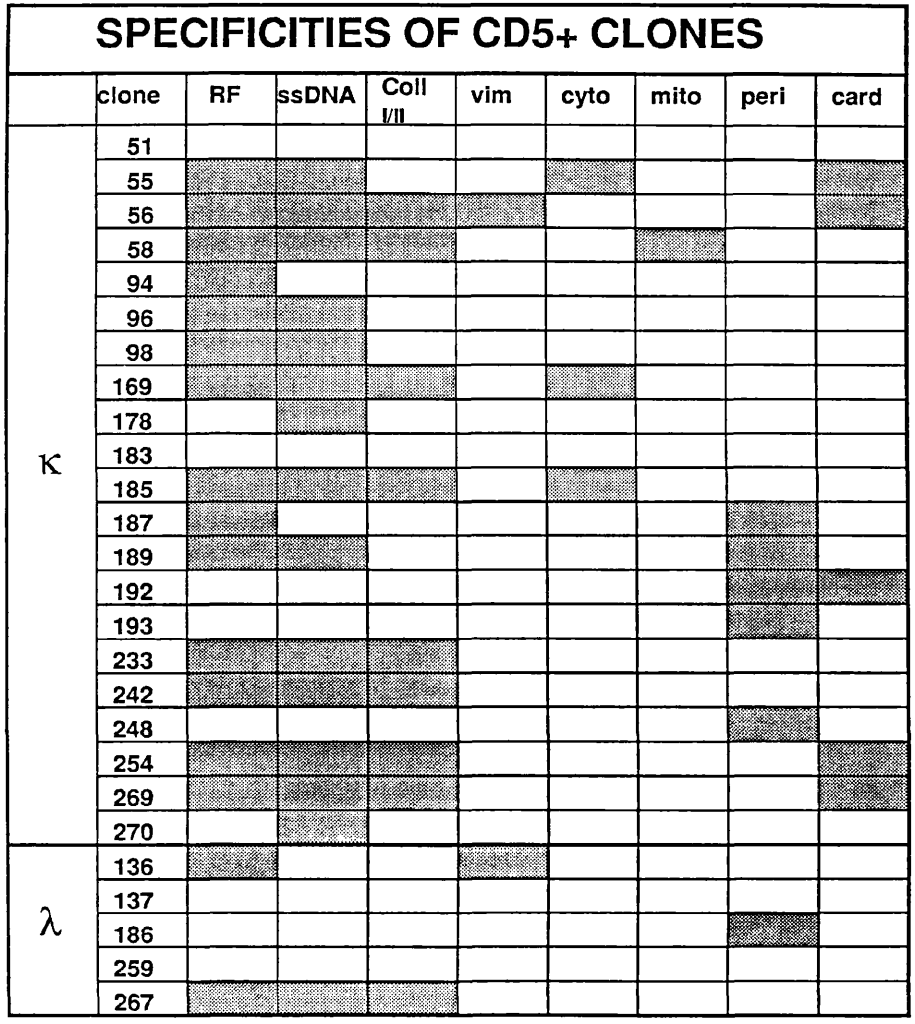
TABLE 2. Summary of antigen specificities of CD5+ and CD5− clones

| Clone (LcH) | RF | ssDNA | RF + ssDNA | ssDNA + coll I/II | ssDNA + Coll I/II | All |
|---|---|---|---|---|---|---|
| CD5+* | | | | | | |
| κ | 31 | 34 | 26 | 15 | 15 | 15 |
| λ | 12 | 10 | 7 | 6 | 5 | 5 |
| CD5−** | | | | | | |
| κ | 9 | 8 | 7 | 4 | 3 | 3 |
| λ | 12 | 10 | 7 | 6 | 5 | 5 |

† Specificity was determined as described in the Materials and Methods.

* 54 κ and 32 λ CD5+ clones and ** 30 κ and 45 λ CD5− clones were analysed

(a)




(b)

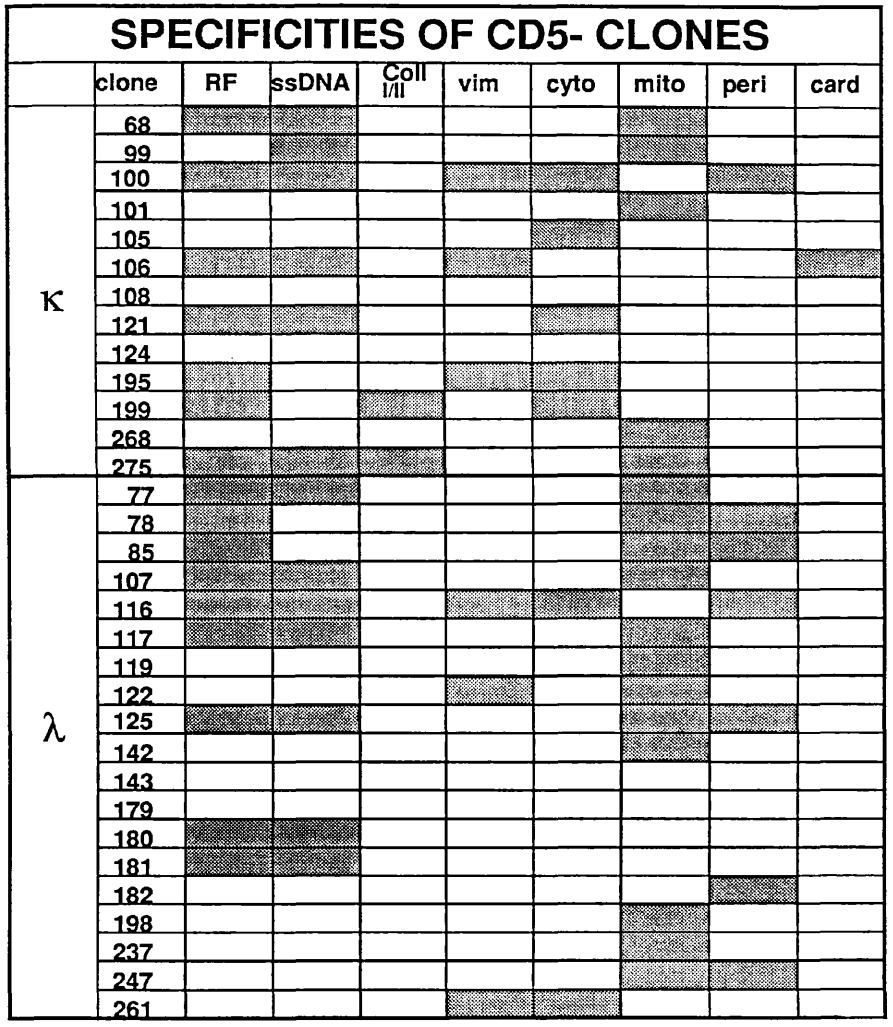



FIG. 2. Specificities of CD5$^+$(a) and CD5$^-$(b) cord blood clones. Key: RF, rheumatoid factor; ssDNA, single-stranded DNA; Coll I/II, collagen type I and type II; vim vimentin; cyto, cytokeratin; mito, mitochondria; peri, perinuclear; card, cardiolipin.

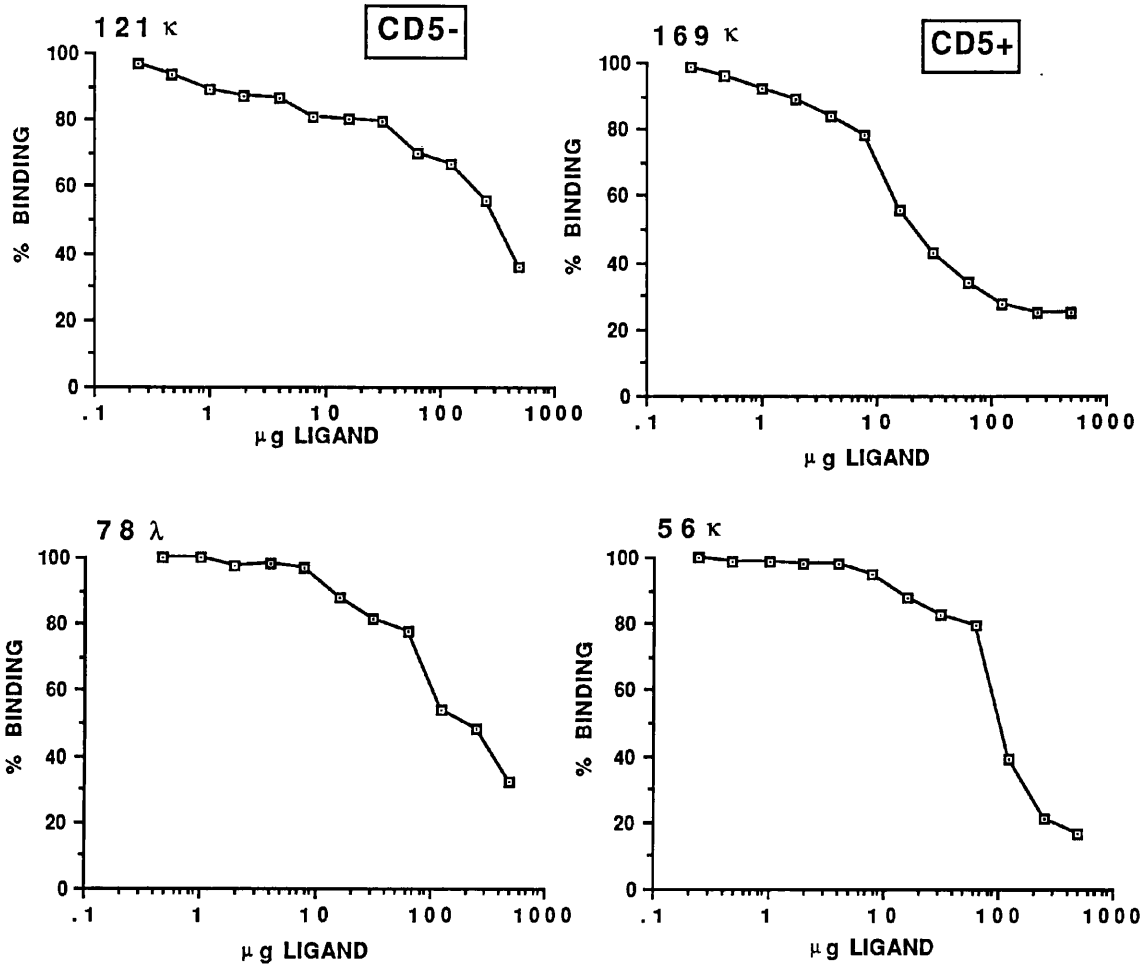



FIG. 3. Blocking of IgM binding from CD5⁺ and CD5⁻ cord blood clones to solid phase IgG with soluble IgG. 2.5 μg IgM antibodies were added to different concentrations of IgG ligand, incubated for 18 h at room temperature and added to IgG-coated ELISA plates as described in the Materials and Methods.

26 CD5⁺ and 32 CD5⁻ clones assayed for the complete autoantigen panel are shown in Fig. 2a and b, respectively. A few CD5⁺ and CD5⁻ B-cell clones also reacted with mitochondria and stained for cytoskeletal elements, confirming the polyreactive nature of many of the clones. In addition a few clones also reacted with golgi and nucleolar (data not shown). No clones produced IgM which bound to thyroglobulin using the coated erythrocyte indicator system in this study.

Monoclonal IgM from both CD5⁺ and CD5⁻ cord blood B cells bound to IgG, ssDNA and collagen I/II in a dose-dependent fashion with similar binding curves (data not shown).

### *Both CD5⁺ and CD5⁻ clones produce IgM antibodies of low avidity*

In general, high concentrations of soluble IgG ligand were required for blocking binding of antibodies to solid-phase IgG. Of eight CD5⁺ and six CD5⁻ clones, most required at least 100 μg/ml of soluble IgG to inhibit 50% of antibody binding, suggesting that they were of low avidity. Two clones from each source are shown in Fig. 3.

## DISCUSSION

The studies presented here demonstrate that the CD5⁺ B lymphocytes detected in relatively large numbers ($\leq 60\%$) in cord blood can be separated as a discrete cell subset from CD5⁻ B cells by FACS and both populations can be induced to secrete immunoglobulin molecules of the IgM isotype by infection with EBV.

At the clonal level, we show that both CD5⁺ and CD5⁻ B cells can be induced to produce IgM antibodies which bind to IgG, ssDNA, collagen types I/II and cytoskeletal components, indicat-

ing that both subjects can potentially produce autoreactive and polyreactive antibodies. Such characteristics have been attributed to those antibodies derived from unimmunized individuals or mice and have been designated 'natural antibodies' [6, 20]. This extends our recent observation that unseparated cord blood and fetal B cells produce IgM antibodies which react with a number of autoantigens [15]. These specificities were found in both $\kappa$ and $\lambda$ and CD5$^+$ and CD5$^-$ clones and binding to a range of autoantigens was shown to be concentration dependent (data not shown). In studies on eight CD5$^+$ and six CD5$^-$ clones, binding of polyreactive IgM antibodies to IgG was only blocked by high concentrations of soluble ligand, suggesting that the avidities of IgM from both sources are low. Two CD5$^+$ and CD5$^-$ clones are shown in Fig. 3.

In this study, the $\kappa$:$\lambda$ ratio was significantly higher in the CD5$^+$ cells than CD5$^-$ cells and is consistent with our previous data [24], indicating that there was not a bias for $\lambda$ light chain expression by CD5$^+$ B cells as there is in the mouse [11].

The *in vitro* system we have used allows us to evaluate the potential antibody repertoire of both the CD5$^+$ and CD5$^-$ B-cell populations without involving activation by antigen. However, there is a possibility of a preferential selection by EBV of IgM producing autoreactive clones in our system. There is evidence that EBV preferentially stimulates B cells early in their differentiation pathway [21] and in the early phase of B-cell activation [4]. Since CD5$^+$ and CD5$^-$ B cells respond to EBV, both populations must contain B cells early in their stage of activation. The frequency of individual cells responding to EBV in the CD5$^+$ versus CD5$^-$ cord blood populations is unclear. The higher frequency of autoreactive CD5$^+$ B cells detected in this study could indicate that there are more B cells primed against endogenous autoantigens within this population than in the CD5$^-$ subset.

Our findings are in contrast with other studies in which the autoantibodies and polyreactive antibodies derived from adult B lymphocytes appear to be an exclusive property of the CD5$^+$ B-cell subset [3,10]. Is there really a fundamental difference between adult and cord blood CD5$^+$ and CD5$^-$ B cells? Our data that clones derived from both CD5$^+$ and CD5$^-$ cord blood B cells can be auto and polyreactive, with similar low affinities and similar specificities to those reported for adult CD5$^+$ B cells [17], would support the notion that at least some CD5$^-$ B cells in the cord blood have similar properties to the CD5$^+$ cord and CD5$^+$ adult B cells. This is supported by the observation that (i) polyreactive and surface CD5$^-$ cord blood EBV B-cell clones can express small quantities of mRNA for CD5 [18], and (ii) about 25% of surface CD5$^-$ cord blood B cells following sorting can be induced by phorbol ester to express some surface CD5 detectable by flow cytometry (unpublished observations). Thus, it might be speculated that it is this particular putative CD5$^-$ B-cell population (perhaps derived from the CD5$^+$ population by loss of surface CD5 expression) which gives rise to auto- and polyreactivity and could explain the lower frequency of these specificities in the cord blood CD5$^-$ population.

Our results indicate that auto and polyreactivity is not restricted to surface CD5$^+$ cord blood B cells. However, it is yet to be established if activation/selection and subsequent loss of the surface CD5 molecule is an ongoing process in the fetal environment. Further studies will determine if these CD5$^+$ cord blood clones utilize immunoglobulin variable region genes differently from some or all of their CD5$^-$ counterparts.

## ACKNOWLEDGMENTS

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# Repertoire of $CD5^+$ and $CD5^-$ cord blood B cells: specificity and expression of $V_H$ I and $V_H$ III associated idiotopes

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## SUMMARY

Epstein–Barr (EBV)-immortalized B cell clones were established from $CD5^+$ and $CD5^-$ cord blood B cells separated by flow cytometry. We have previously shown that IgM from many of the clones was polyreactive, exhibiting reactivity with a number of autoantigens. In this study, IgM produced by the clones was analysed by MoAb for the expression of cross-reactive idiotypes (CRI) associated with rheumatoid factor paraproteins and from defined $V_H$ and $V_\kappa$ subgroups of immunoglobulin heavy and light chains. IgM produced by clones established from $CD5^+$ and $CD5^-$ B cells expressed the $V_H$ I associated idiotope G8. Furthermore, IgM produced by both sets of clones exhibited a similar frequency of $V_H$ III heavy chain subgroup expression, as determined by reactivity with staphylococcal protein A (SpA) and $V_H$ III-associated CRI expression (B6 and/or D12). In contrast, expression of the $V_\kappa$ III-associated 17.109 CRI was significantly higher in IgM antibodies produced by clones established from $CD5^+$ compared with the $CD5^-$ clones (32 *versus* 5%: $P < 0{\cdot}05$). Analysis of the $V_H$ and $V_L$ subgroup expression by IgM produced by the $CD5^+$ and $CD5^-$ cord blood clones, and their autoantigen reactivity profile did not reveal restriction or selection within $CD5^+$ and $CD5^-$ populations. However, our data suggest that differences may exist in the expression of certain germ-line genes between $CD5^+$ and $CD5^-$ cord blood B cells and might indicate an expansion of $CD5^+$ B cells within the fetal environment.

**Keywords** $CD5^+$ B cells cross-reactive idiotypes cord blood B cells B cell repertoire

## INTRODUCTION

A subpopulation of adult human B cells expressing the 67-kD molecule CD5 has been shown to be the major subset for the production of anti-ssDNA and anti-IgG Fc (rheumatoid factor, RF) [1,2]. Furthermore, this population has been shown to produce low-avidity polyreactive antibodies [3]. Malignant B cells from most patients with chronic lymphocytic leukaemia also carry CD5 and show high frequency of auto- and polyreactivity revealed by *in vitro* activation with phorbol esters [4,5].

We have previously shown that cord blood B cells, rich in a CD5-expressing B cell population, produce IgM antibodies which exhibit auto and polyreactivity after immortalization by Epstein–Barr virus (EBV) [6]. Furthermore, clones derived from both pre-sorted $CD5^+$ and $CD5^-$ cord blood cells exhibit auto- and polyreactivity with a higher frequency of anti-IgG Fc and ssDNA activity in the $CD5^+$ population [7].

A number of studies in mice have suggested that $CD5^+$ B cells might differ in the expression of variable region genes from their conventional counterparts [8–10]. Although the definitive data for V gene usage must come from sequence analysis of rearranged and transcribed immunoglobulin genes, in this study we have examined the clonal expression of $V_\kappa$, $V_H$ III and $V_H$ I subgroups as determined by their expression of certain CRI. A number of studies have suggested that these CRI are expressed by germ-line or minimally mutated germ-line genes [11]. CRI studied here have previously been shown to be expressed in unsorted clones derived from cord blood B cells [12].

In the present study, EBV immortalized cell clones were established from sorted $CD5^+$ and $CD5^-$ cord blood B cells. Our data show a preference of the $V_\kappa$ III sub-subgroup associated CRI 17.109 with the clones established from the $CD5^+$ population. A similar frequency of the $V_H$ I and $V_H$ III associated CRI was observed with clones established from both $CD5^+$ and $CD5^-$ populations.

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## MATERIALS AND METHODS

*Preparation of $CD5^+$ and $CD5^-$ clones*

Cord blood cells from three neonates were stained with CD5 (Leu 1) and CD20 (Leu 16) MoAb and sorted into $CD5^+$ and

$CD5^-$ cells by a FACScan flow cytometer (Becton Dickinson) as previously described [7]. Clones were derived by immortalization with EBV and shown to be monoclonal by limiting dilution analysis and light chain restriction using an ELISA, as previously reported [6]. Additional evidence for monoclonality comes from the expression of single heavy chain isotypes and CRI of each subgroup and reactivity with SpA. Supernatants from clones containing >500 ng/ml of IgM were used for analysis.

*Determination of autoantibody specificities*

The antibody specificities for a panel of autoantigens were detected as previously described [4,6,7]. Briefly, antibodies to the Fc of IgG (rheumatoid factor), collagen types I and II, ssDNA, cardiolipin and histones were detected by an ELISA. Antibodies to tubulin, actin, vimentin, golgi and cytokeratin were detected in a cell line IMR-33 derived from a gerbil fibrosarcoma (American Tissue Type Culture Collection, Rockville, MD) as previously reported [13]. Antibodies to vimentin were confirmed by the perinuclear pattern of staining following colchicine pretreatment. Anti-nuclear (ANA) and nucleolar antibodies were detected by indirect immunofluorescence using HEP-2 cells [13]. Anti perinuclear antibodies were detected by immunofluorescence using human buccal cells as previously described [14].

*Detection of $V_H$ III clones by staphyloccocal protein A (SpA)*

IgM was tested by ELISA and reverse passive haemagglutination assay for binding to SpA as described elsewhere [15,16]. ELISA for SpA binding were performed using polystyrene microtitre plates sensitized with 10 $\mu$g/ml SpA by incubation at 37°C for 2 h. Supernatants from established EBV clones were added and incubated for a further 2 h at 37°C. Bound IgM was revealed with horseradish peroxidase (HRP) conjugated sheep anti-human IgM (Binding Site, Birmingham, UK). Reverse passive haemagglutination assay was performed using sheep red blood cells sensitized with SpA using chromic chloride [17]. Supernatants containing immunoglobulins were titrated in HEPES-RPMI) supplemented with 2% FCS, 30 $\mu$l of SpA sensitized cells added and the haemagglutination results recorded after 2 h incubation at room temperature.

*Preparation of idiotope reagents*

Immunization and fusion protocols, using the murine myeloma line NSO as a fusion partner, have been described in detail elsewhere [18,19]. MoAb with specificity to idiotypes and light chain subgroup determinants were produced to two IgM RF paraproteins; Ko ($V_\kappa$ IIIb/$V_H$ I) from the Wa cross-reactive idiotype family and He ($V_\kappa$ III/$V_H$ III) as previously described [18–20,21]. The immunoglobulin heavy and light chain V gene family and major cross-reactive idiotype (CRI) associations of immunoglobulins recognized by the monoclonal reagents were characterized by ELISA, haemagglutination and Western blot analyses [18,19,22]. MoAb G6 and G8 recognize $V_H$ I associated idiotopes, B6/D12 are associated with the $V_H$ III family. C7 MoAb recognizes a $V_\kappa$ III sub-group framework determinant and C6 a $V_k$ IIIb sub-subgroup frame work determinant.

MoAb 17.109 (mouse IgG2b) was produced to IgM RF paraprotein Sie [23]; $V_\kappa$ IIIb/VH I), from the Wa CRI family, and kindly provided by Dr D. Carson (Research Institute of Scripps Clinic, La Jolla, USA). This MoAb recognizes a $V_\kappa$ IIIb sub-subgroup associated idiotope.

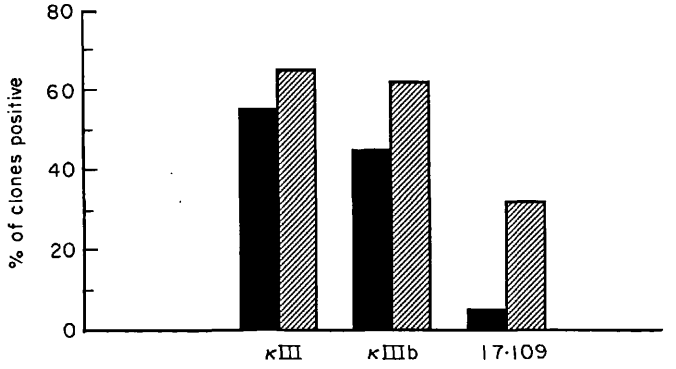


**Fig. 1.** More frequent expression of the $V_\kappa$ IIIb sub-subgroup associated idiotope 17.109 by $CD5^+$ than $CD5^-$ kappa clones. $P<0{\cdot}025$. $V_\kappa$ III subgroup and $V_\kappa$ IIIb sub-subgroup associated isotypic determinants were detected using an ELISA with MoAb C7 and C6 respectively. The $V_\kappa$ IIIb sub-subgroup associated CRI 17.109 was also detected in this way. IgM from 20 $CD5^-$ and 34 $CD5^+$ B cell clones was examined.

*Detection of idiotopes*

Expression of CRI by IgM from the $CD5^+$ and $CD5^-$ derived cord blood clones was determined as previously described [12,24]. Briefly, microtitre ELISA plates (Flow, UK) were sensitized with sheep anti-human IgM at 10 $\mu$g/ml by incubation at 37°C for 2 h. Culture supernatants were added to the sensitized wells and incubated for 2 h at 37°C in a humidified incubator with 5% $CO_2$. Monoclonal anti-light chain and heavy chain isotypes, CRI and subgroups were added and incubated for 2 h at 37°C. All MoAb were used at 1/500 dilution of the ascites. Bound murine MoAb were revealed with HRP-conjugated sheep anti-mouse IgG (Binding Site). Background values of wells sensitized with control MoAb and HRP-conjugated sheep anti-human $\mu$ chain, were automatically subtracted and values significantly higher than the negative controls were considered to be positive. IgM expressing CRI gave OD values >0·5 OD units at 492 nm compared with background values of less than 0·05. Monoclonal anti-$\mu$, $\kappa$ and $\lambda$ light chains were clones AF6, 6e1 and C4 respectively (Oxoid-Unipath, Bedford, UK).

## RESULTS

*More frequent use of kappa light chains and $V_\kappa$ IIIb sub-subgroup associated idiotope 17-109 by $CD5^+$ B cells*

Of 86 clones derived from $CD5^+$ B cells, 54 used $\kappa$ chains (63%) while significantly fewer, 30 of 75 (40%) $CD5^-$ B cells clones used this light chain isotype ($P<0{\cdot}01$: data not shown).

In relation to the $\kappa$ clones studied, the frequency of the $V_\kappa$ III sub-group and the $V_\kappa$ IIIb sub-subgroup both $CD5^+$ and $CD5^-$ clones was similar (Fig. 1). In both groups the $V_\kappa$ IIIb sub-subgroup represented >75% of the $V_\kappa$ III family chains used, as detected using the MoAb C6. However, more of the $CD5^+$ clones (11 of 34) expressed the $V_\kappa$ IIIb sub-subgroup associated CRI 17.109 ($P<0{\cdot}025$) than the $CD5^-$ clones (1 of 20).

*Expression of the $V_H$ I associated idiotopes G6 and G8 by $CD5^+$ and $CD5^-$ clones*

IgM from none of the 40 $CD5^+$ and 45 $CD5^-$ clones examined expressed the $V_H$ I associated idiotope G6. However, IgM from

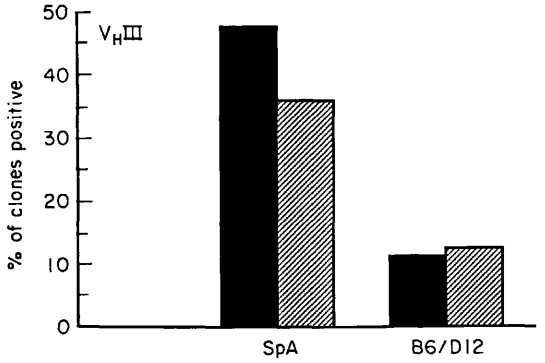


**Fig. 2.** Similar frequency of $V_H$ I and $V_H$ III associated CRI expressed by IgM from CD5$^+$ and CD5$^-$ clones. G8 (a $V_H$ I associated CRI) and B6 and D12 ($V_H$ III associated CRI) MoAb were used in ELISA as described in Materials and Methods. IgM from 45 CD5$^-$ and 40 CD5$^+$ clones was examined. IgM from 46 CD5$^-$ and 47 CD5$^+$ B cell clones was analysed for binding to SpA as an indication of $V_H$ III usage.

**Table 1.** Autoantibody specificities of CD5$^+$ and CD5$^-$ cord blood clones using $V_H$ I and $V_H$ III family idiotopes

| | CD5$^+$ | | | CD5$^-$ | | |
|---|---|---|---|---|---|---|
| | | $V_H$ III | | | $V_H$ III | |
| Specificity | $V_H$ I G8 | SpA | B6/D12 | $V_H$ I G8 | SpA | B6/D12 |
| Fc | 1/2 | 8/15 | 3/4 | 2/3 | 6/21 | 3/5 |
| ssDNA | 1/2 | 6/15 | 3/4 | 2/3 | 4/21 | 1/5 |
| Polyreactive $\geqslant$2 antigens | 1/2 | 6/15 | 3/4 | 3/3 | 8/21 | 4/5 |

Summary of specificities of antibodies from the different clones for Fc of human IgG, ssDNA and two or more autoantigens (polyreactive). Note that clones utilizing either of the two $V_H$ families reacted with these autoantigens and were polyreactive. Specificities for different autoantigens were determined as described in Materials and Methods.

two CD5$^+$ and two CD5$^-$ $\kappa$ clones expressed the idiotope G8. All four of these G8 positive clones were associated with kappa chains using $V_\kappa$ III family genes and two were $V_\kappa$ IIIb positive (data not shown). One $\lambda$ clone expressing G8 was detected in the CD5$^-$ group.

*Equal representation of $V_H$ III gene family use and expression of B6 and D12 CRI in CD5$^+$ and CD5$^-$ clones*

IgM from more CD5$^-$ (22/46) than CD5$^+$ clones (16/47) bound SpA but this was not significant at the 5% level (Fig. 2). This suggested similar usage of $V_H$ III family genes by clones of CD5$^+$ and CD5$^-$ origin. Similar frequencies of SpA$^+$, $\kappa$ and $\lambda$ light chain expressing clones were also seen in both populations. The $V_H$III associated idiotopes B6 and/or D12 were also present on IgM at the same frequency in both CD5$^+$ and CD5$^-$ populations (Fig. 2). Both these CRI's were co-expressed by the same clones in all cases except 3$\lambda$ CD5-clones which were B6 negative but D12 positive (data not shown).

*Specificity of clones and their CRI expression*

Many of the clones in this study have previously been shown to be polyreactive [7]. One of the two CD5$^+$ $\kappa$ light chain expressing clones which used $V_\kappa$ IIIb together with the $V_H$ I associated CRI, G8 was polyreactive, reacting with Fc, ssDNA and perinuclear antigen. The other $\kappa$ clone did not react with any autoantigen in the panel tested. One of the G8 positive, CD5$^-$ clones which expressed $V_\kappa$ III was polyreactive whilst the other used $V_\kappa$ IIIb subgroup of light chain and reacted with ssDNA and mitochondria. Only the CD5$^-$ $\lambda$ clone expressing G8 was also polyreactive. Thus four out of five of the G8 expressing clones were autoreactive (Table 1).

IgM from similar percentages of CD5$^+$ and CD5$^-$ clones utilizing $V_H$ III family associated genes as determined by SpA binding showed auto and polyreactivity to the same degree (Table 1). This was also seen for the $V_H$ III associated CRI B6 and D12 (Table 1).

## DISCUSSION

In this study we demonstrate that IgM produced by EBV established CD5$^+$ and CD5$^-$ B cell clones express the $V_H$ I associated CRI, G8, bind SpA and express the $V_H$ III associated CRI B6 and D12 at a similar frequency. There was, however, a significant increase in the frequency of the 17.109 CRI expression on IgM produced by CD5$^+$ clones compared with the CD5$^-$ clones.

*Expression of $V_H$ I associated idiotopes*

The $V_H$I associated CRI G8 was found on IgM produced by both CD5$^+$ and CD5$^-$ B cell clones at a similar frequency, 5 and 7% respectively. The majority, (4/5) clones, displayed auto and polyreactivity. Interestingly, the $V_H$ I associated idiotope G6 was not found on IgM produced by either the CD5$^+$ or CD5$^-$ clones in this study, confirming our previous data on clones obtained from unsorted cord blood B cells [12]. Immunohistological studies using tissue sections of fetal spleen have identified small clusters of G6$^+$ B cells in the primary follicles [25]. The reason for this discrepancy is unclear but might indicate the inability of G6 positive B cells to be immortalized by EBV. Alternatively, since all the clones in the study were derived from only three cord blood samples, this may indicate a polymorphism in the expression of this CRI within the normal cord blood population. Indeed, B lymphocytes from 10% of adults fail to express G6 (T. J. Kipps, personal communication). Our recent findings [12] and those of others [26] that malignant B cells from a high frequency of CLL patients clones express G6 indicates that at least CD5$^+$ clones immortalized by oncogenic events can express G6.

*Expression of $V_H$ III family associated products*

Previous studies by others [15] have shown that IgM binding to SpA is a characteristic of the $V_H$ III family of paraproteins. Forty-eight per cent of the CD5$^+$ and 55% of the CD5$^-$ clones were SpA$^+$ and therefore it is likely that these clones use the $V_H$ III family of Ig heavy chains. The slightly lower proportion of CD5$^+$ clones utilizing this family of heavy chain genes probably reflects the higher frequency of $V_H$ IV associated idiotopes found in the CD5$^+$ population [27]. The high percentages of both CD5$^+$ and CD5$^-$ clones expressing $V_H$ III family of genes is consistent with the high percentage of polyclonal IgM using $V_H$ III in the serum of adults [21]. In fact, the $V_H$ III family is the largest of the $V_H$ families in man, containing at least 100 genes and probably comprises many gene subfamilies [28].

The $V_H$ III associated CRI recognized by the monoclonal B6 is fairly common amongst SpA$^+$ clones (about 25%) and is equally represented amongst the CD5$^+$ and CD5$^-$ clones. This B6 CRI was first identified on an IgM paraprotein He with anti-IgG-Fc specificity and appears from blocking studies to be present in or close to the antibody binding site [16,21]. As previously concluded from studies with cord blood clones not separated on the basis of CD5$^+$ and CD5$^-$ origin [12] B6 positive IgM was not exclusively specific for IgG-Fc. Some clones reacted with mitochondria and vimentin only while others were polyreactive. The B6 monoclonal antibody apparently reacts with a CRI which is probably encoded by a limited number of $V_H$ III genes and therefore suggests that even though the $V_H$ III family is the largest $V_H$ family [28], there is some restriction in the use of these genes early in ontogeny. The idiotope recognized by the D12 antibody had a similar distribution on both CD5$^+$ and CD5$^-$ clones. It is interesting that this CRI, although frequently expressed on the same clones as B6, was found in few instances without B6 expression on IgM from some clones. This may be indicative of genes encoding this protein being of some major functional importance. Analysis of the antibody specificities of the $V_H$ III expressing clones showed that auto and polyreactivity could be achieved using either $\kappa$ or $\lambda$ chains (data not shown).

*Expression of $V_\kappa$ family sub-groups and associated idiotopes*

The frequency of $\kappa$ light chain expressing clones was highest in those derived from the CD5$^+$ clones as previously reported [7, 27]. The majority of the clones described in this study have previously been shown to be auto and polyreactive [7]. In most cases, the frequency of IgM$\kappa$ expressing clones which were auto and polyreactive to the panel of antigens tested, was higher than the $\lambda$ light chain bearing clones in both CD5$^+$ and CD5$^-$ groups. However, this was only significant within the CD5$^+$ population where 34/54 $\kappa$ clones compared with 10/32 $\lambda$ expressing clones produced IgM with specificity for ssDNA ($P < 0{\cdot}025$).

Whereas IgM from both B cell populations expressed high levels of the $V_\kappa$ IIIb sub-group, significantly more of the CD5$^+$ clones (32%) reacted with the 17.109 monoclonal antibody than the CD5$^-$ clones (5%). This antibody recognizes the product of the germ-line gene $V_\kappa$ 325 which is associated with the $V_\kappa$ IIIb sub-group of light chain [29]. 17.109 positivity has been shown to be associated often with $V_H$ I in around 20% of B CLL patients [26]. Other studies have demonstrated that at least 7% of normal fetal spleen cells express the 17.109 CRI and some of these appeared to be associated with heavy chain genes from the $V_H$ I family [25]. In the study presented here, none of the 17.109 positive clones were associated with either G6 or G8 idiotopes ($V_H$ I family). In a previous study, 17.109 was found to be frequently associated with G6 in paraproteins with RF activity [30]. Three of the 17.109$^+$ clones used $V_H$ III subgroup of heavy chain. The other three 17.109 positive clones were associated with $V_H$ IV subgroup of heavy chain [27], products of which are encoded by only a small number ($\leqslant 10$) of genes [31]. Of the 17.109$^+$ clones, all but one produced IgM which was autoreactive (anti-ssDNA and anti Fc). Seven of them were also polyreactive (data not shown).

The similarity of specificities of the CD5$^+$ and CD5$^-$ cord blood clones [7] together with similar expression of $V_H$ I and $V_H$ III associated idiotopes in this study indicates that many of the CD5$^-$ cord blood clones have characteristics of the CD5$^+$ cord blood and adult B cells [1,2]. An interesting possibility is that many of the CD5$^-$ cord blood B cells may represent the human homologue of the murine 'sister population' which has been shown to produce autoantibodies [32]. A similar 'Sister population' has recently been described in adult human blood (Casali, personal communication). The relatively high frequency of CD5$^+$ clones expressing 17.109 sub-subgroup CRI compared with the CD5$^-$ clones shown here, together with the exclusive expression of a $V_H$ IV associated idiotope by some of the CD5$^+$ clones [27] is consistent with the selective expansion of CD5$^+$ cells during development. That cord blood B cells might be activated *in vivo* is suggested by the high frequency of these cells expressing the activation marker CD78 (F. Yuksel *et al.*, unpublished observations). The nature of such a stimulus could be fetal auto-antigens which include IgM idiotypes. Such idiotypic 'connectivity' has been described in the mouse [33] and could be involved in setting up the idiotype network during normal development. Further studies will focus on potential connectivity of the CD5$^+$ B cells and sequence analysis of these clones will provide information as to the mechanism by which they arise.

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# Selective Expression of a $V_H$IV Subfamily of Immunoglobulin Genes in Human CD5$^+$ B Lymphocytes from Cord Blood

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## Summary

Human B lymphocytes expressing the CD5 surface antigen (CD5$^+$ B cells) constitute a subset capable of producing polyspecific antibodies recognizing a variety of self antigens. The repertoire of antibodies produced by CD5$^+$ and CD5$^-$ B cells is different. However, it is not yet established whether this distribution is reflected in different immunoglobulin variable region gene (IgV) use. Rearrangement of heavy chain IgV ($IgV_H$) genes represents one of the first identifiable stages in the maturation of B cells, and occurs in a developmentally ordered fashion. The repertoire of $IgV_H$ gene expression is highly restricted during fetal life but diversifies progressively after birth. A high frequency of $V_H$ gene use from the relatively small $V_H$IV gene family has previously been demonstrated in human fetal liver B cells. In the present study, 102 B cell lines established by Epstein-Barr Virus-transformation of separated CD5$^+$ and CD5$^-$ cord blood B cells, were examined for the frequency of IgV expression using monoclonal antibodies to cross-reactive idiotypes (CRI). The results demonstrate a relatively high frequency of $V_H$IV gene use (30%) in B cells from cord blood. Furthermore, two mutually exclusive CRI associated with distinct subgroups of the $V_H$IV family are segregated in their association with either subset of B cells. One CRI is exclusively expressed in lines established from CD5$^+$ B cells while the other is associated with lines established from CD5$^-$ B cells.

During the past few years, heterogeneity within B lymphocyte populations has become increasingly apparent. Several studies have demonstrated functional, physiological, and developmental heterogeneity. Studies in the mouse suggested that two separate lineages of B cells may exist (1). One lineage, is defined by cell surface expression of the Ly-1 antigen, characteristic of T lymphocytes, and the secretion of polyspecific antibodies reactive with a variety of self antigens. The second lineage lacks the Ly-1 antigen and produces antibodies, mainly, to exogenous antigens (1). It is not clear, however, if the distinction is also reflected in antibody variable region gene use by the Ly-1$^+$ and Ly-1$^-$ B cell subsets. This question can be approached by analysis of variable region gene segment rearrangements, at the DNA level or through serological markers expressed on the protein products.

In humans, the majority of B lymphocytes in fetal liver, cord blood, and a minority in adult peripheral blood and lymph nodes express surface CD5 molecule, the human equivalent of mouse Ly-1 (2).

In the present study, we have used a number of mAbs that recognize cross-reactive idiotypes (CRI)[1] associated with individual $V_H$ or $V_L$ gene family products to examine IgV gene use in CD5$^+$ and CD5$^-$ B lymphocytes.

## Materials and Methods

*Preparation and Sorting of B Lymphocytes.* B Lymphocytes were enriched from heparinized blood of three full-term donors by centrifugation over Ficoll-Hypaque and rosetting using SRBCs (3). The enriched B cell populations were stained simultaneously with fluorescein-conjugated anti-CD20 (clone Leu-16) and PE-labeled anti-CD5 (clone Leu-1) and sorted using a FACStar® (Becton Dickinson Immunocytometric Systems, Mountain View, CA). Lymphocytes displaying green fluorescence only (CD20$^+$, CD5$^-$ cells) and those displaying green and red fluorescence (CD20$^+$, CD5$^+$ cells) were gated and sorted as CD5$^-$ and CD5$^+$ B cells, respectively.

[1] *Abbreviation used in this paper:* CRI, cross-reactive idiotypes.


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The degree of cell separation was evaluated by re-analysis of the PE-labeled cells by FACSCAN (Becton Dickinson and Co.).

*Establishment of Epstein-Barr Virus Transformed Cell Lines.* Sorted $CD5^+$ and $CD5^-$ B cells were infected with EBV using supernatant from the B95-8 marmoset line, and cultured in the presence of $2 \times 10^4$ irradiated (40 Gy) PBMCs (3). After culture for 2–7 wk, supernatants were harvested and assayed for the presence of Ig using an ELISA. Culture plates with <33% of wells positive for growth and immunoglobulin secretion were further evaluated for the clonality of the Ig product using antibodies specific for the IgM, IgG, IgA, $\kappa$, and $\lambda$ isotypes. Cell lines positive for a single heavy and light chain isotype only were investigated for the expression of $V_H$I (G6 and G8), $V_H$III (B6 and D12), and $V_H$IV (LC1, R2.1A2, and 9G4) heavy chain subgroup associated CRI.

*Production of Murine Monoclonal Antibodies.* The production and specificity of all mAbs used has been reported (4–10). Antibodies C7 and 17-109 have specificity for proteins expressing $V_H$III subgroup and a $V_K$IIIb sub-subgroup associated CRI, respectively (5, 6). LC1 (kindly provided by Dr. C. Winearls and Miss D. Brennan, Hammersmith Hospital, London, UK) recognizes a CRI associated with the $V_H$IV of an IgM rheumatoid factor paraprotein (7); R2.1A2 (9), and 9G4 (10) specific for CRI expressed on 90% of IgM paraproteins with cold agglutinin activity. Mab 9G4 was raised in a Lou rat.

*Human Monoclonal Paraproteins and Defined B Cell Clones.* Paraproteins with cold agglutinin activity (from patients ODO and HAW) were isolated from the serum of patients with cold agglutinin disease, and specificity established using adult human $O^+$ red blood cells at 4½ (10). Rheumatoid factor paraproteins (from patients DIN and COR) were isolated from the serum of patients having essential mixed cryoglobulinemia by affinity chromatography. Paraproteins of unknown specificity were isolated from the serum of patients with Waldenstrom's macroglobulinemia by Sephacryl-S200 gel filtration (Pharmacia, Uppsala, Sweden). Proteins from patients ODO, DIN, COR, STR, and CAL were typed, serologically, for expression of heavy and light chain subgroups (11) and kindly provided by Dr. Gregg Silverman (Research Institute of Scripps Clinic, La Jolla, CA). Supernatants from the B cell clones AB26, AB17 and AB44 were a kind gift from Dr. Don Capra (Southwestern Medical Center, University of Texas, Dallas, TX). The clones were established by EBV transformation of sorted $CD5^+$ B lymphocytes from the peripheral blood of a normal individual. The variable region subgroup utilized was determined by cDNA sequencing (12).

*Enzyme-Linked Immunosorbent Assay (ELISA).* Microtitre ELISA plates (Linbro, Flow Labs, High Wycombe, Bucks, UK), sensitized with polyclonal sheep anti-human $\mu$ chain were used to bind IgM in the undiluted culture supernatant of B cell lines. Purified proteins positive or negative for the expression of the probed heavy and light chain isotypes and CRI were included in each assay to confirm specificity. Following three washes with PBS containing 0.05% Tween 20 (PBS/T), murine mAb with specificity for $\mu$, $\kappa$, and $\lambda$, (clones AF6, 6e1, and C4, respectively; Unipath-Oxoid, Bedford, UK) or CRI were added at 1/500 dilutions in PBS/T. Bound mAb were revealed with horseradish peroxidase conjugated sheep anti-mouse or anti-rat Igs (Binding Site Limited, Birmingham, UK). Plates were read using a Titretek Multiscan reader (Flow Labs, High Wycombe, Bucks, UK) and OD value recorded following subtraction of background control values. The sensitivity of the assays allowed detection of antigen at a concentration of 5–10 ng/ml. Since Ig levels in the supernatants of the EBV lines were 500–5,000 ng/ml the assays provide further evidence suggestive of monoclonality and all profiles obtained were consistent with the establishment of clones.

*Statistical Analysis.* The expression of CRI were compared in $2 \times 2$ tables using the chi-squared test ($\chi^2$) or Fisher's two-tailed test depending on the number of samples examined.

## Results and Discussion

Following infection with EBV and culture for 2–7 wk, plates with <33% of wells positive for cell growth were selected for study of the expression of CRI. This cut off point provides for a statistical probability of monoclonality on the basis of Poisson analysis of limiting dilution protocols (13). Additionally, the criterion of expression of a single light and heavy chain isotype was observed for all cell lines studied. Furthermore, none of the cell lines expressed CRI from more than one $V_H$ subgroup of heavy chains. The sensitivity of the ELISA allowed for the detection of Ig produced by <1% of the lymphocytes present; assuming they all have the same capacity to produce Ig. Thus, while monoclonality is not formally proven, all findings are consistent with such a conclusion. By these criteria a total of 102 cell lines were established by EBV transformation of cord blood lymphocytes—53 from the $CD5^+$ population of B cells and 49 from the $CD5^-$ population. Thirty-six of the 53 $CD5^+$ cell lines expressed K light chain, while 17 expressed $\lambda$ light chain, a $\kappa/\lambda$ ratio of 2:1, roughly the ratio found in circulating Igs. For the $CD5^-$ cell lines a considerably lower $\kappa/\lambda$ ratio of 1:1.6 was observed.

Previous studies demonstrated a relatively high frequency of $V_K$III subgroup expression in B cells from patients with chronic lymphocytic leukaemia which express surface CD5 (14). Within our K-expressing $CD5^+$ lines 12/36 (30%) expressed the $V_K$III subgroup of light chain (Table 1), a frequency similar to the expected frequency of this light chain

**Table 1.** *$V_K$III Subgroup Expression within Cell Lines Established from $CD5^+$ or $CD5^-$ B Lymphocytes and Association with 17-109 CRI Expression*

| | Lines expressing $V_K$III | | Lines expressing 17-109 | | |
|---|---|---|---|---|---|
| Cell line origin | Total | % of K lines | Total | % of $V_K$III lines | % of K lines |
| $CD5^+$ | 12 | 30 | 8 | 67 | 23 |
| $CD5^-$ | 5 | 26 | 1 | 20 | 5 |

**Table 2.** *Reactivity of Monoclonal Antibodies Specific for $V_HIV$ Associated CRI with Proteins from the $V_HIV$ Family of Genes*

| Protein | Isotype $V_H$-$V_L$ subgroup | Specificity | Reactivity with anti-$V_H$IV CRI | | |
|---|---|---|---|---|---|
| | | | LC1 | R2.1A2 | 9G4 |
| DIN | IgM-$V_H$IV $V_K$III | Rheumatoid factor | + | − | − |
| COR | IgM-$V_H$IV $V_K$III | Rheumatoid factor | + | − | − |
| STR | IgM-$V_H$IV $V_K$III | Unknown | + | − | − |
| CAL | IgM-$V_H$IV $V_K$II | Unknown | + | − | − |
| ODO | IgM-$V_H$IV $V_K$III | Cold agglutinin | − | + | + |
| HAW | IgM-$V_H$IV $V_K$II | Cold agglutinin | − | + | + |
| AB26 | IgM-$V_H$IV $V_\lambda$ | Polyreactive | + | − | − |
| AB17 | IgG3-$V_H$IV $V_K$III | Polyreactive | − | + | + |
| AB44 | IgA-$V_H$IV $V_\lambda$I | Polyreactive | − | + | + |

Reactivity of monoclonal anti-CRI was determined in ELISA. $V_H$ and $V_K$ subgroup of proteins DIN, COR, STR, CAL, ODO, and HAW were assigned by immunoblot analysis with peptide induced reagents. Expression of $V_K$III subgroup was confirmed by reactivity with monoclonal antibody C7. $V_H$ and $V_L$ of proteins AB26, AB17, and AB44 were assigned by DNA sequence analysis.

subgroup in polyclonal IgM. The proportion of $V_K$III in the K-expressing CD5$^-$ lines was similar (26%) (Table 1). However, the frequency of $V_K$III light chains expressing the 17-109 CRI was higher (Fisher's two-tailed probability = 0.13) in the CD5$^+$ lines compared with the CD5$^-$ ones.

$V_H$ gene usage within the cell lines was investigated using a panel of mAbs specific for $V_H$ subgroup associated CRI. The significant finding was expression of $V_H$IV–associated CRI. This is of particular interest because recent studies by Sanz et al. (12) have demonstrated a remarkably high frequency of $V_H$IV gene use in B cell clones established by EBV transformation of CD5$^+$ B cells from the peripheral blood of normal individuals. Furthermore, due to the small number of genes within this family (~10) and the high degree of internal homology (at least 91.5% between the most distant members to a consensus $V_H$IV sequence) it is probable that the germline gene repertoire would be represented by relatively few CRI.

In this study we applied three anti-CRI reagents, two of which appear to recognize the same or mutually inclusive CRI (R2.1A2 and 9G4). The third reagent (LC1) recognizes a CRI that is mutually exclusive to R2.1A2 and 9G4 (Table 2). Although neither CRI could be formally assigned to reported $V_H$IV genes, association with some germline genes is possible, on the basis of the present studies and recent studies of cold agglutinins (11) and polyreactive antibodies (12). Thus, the CRI recognized by LC1 may be a phenotypic marker for the 71-2 and 71-4 genes and related genes, whereas R2.1A2/9G4 may be markers for the 4-21 and related genes.

Our analyses reveal that: (*a*) The representation of $V_H$IV genes in these B cell lines is considerably higher than would be predicted for this relatively small family of genes. (*b*) A relatively higher but insignificant proportion of CD5$^+$ cell lines express $V_H$IV genes compared to their CD5$^-$ counterparts (35% vs. 28%; $\chi^2 = 0.62$, $p > 0.1$). (*c*) Remarkably, whereas 13/53 (24%) cell lines derived from the CD5$^+$ population coexpressed both R2.1A2 and 9G4, no CD5$^-$ cell lines expressed either of these CRI (Table 3) ($\chi^2 = 13.8$, $p < 0.001$). (*d*) Conversely, 14/49 (28%) CD5$^-$ lines expressed LC1, while only 6/53 (11%) CD5$^+$ lines expressed this CRI ($\chi^2 = 4.8$, $p < 0.05$).

Thus, it appears that a significant proportion of CD5$^+$ B cell lines produce IgM derived from a single gene, or a small number of genes within the $V_H$IV family for which a CRI associated with anti-I-carbohydrate antigen specificity is a phenotypic marker. Both R2.1A2 and 9G4 were coexpressed on the same molecules and were associated with $\kappa$ light chain expression in 9/13 (70%) of the cell lines. However, none of the cell lines could be demonstrated to express the $V_K$III epitope C7 or 17-109 together with R2.1A2 and 9G4. This appears to be in contrast to the observed association of $V_H$IV heavy chains and $V_K$III light chains in cold agglutinins (15).

**Table 3.** *$V_HIV$–associated CRI Expression in CD5$^+$ and CD5$^-$ Cell Lines Established from Cord Blood Lymphocytes*

| Cell line origin | Total no. | No. (%) lines expressing | | |
|---|---|---|---|---|
| | | LC1 | R2.1A2 | 9G4 |
| CD5$^+$ | | | | |
| IgM $\kappa$ | 36 | 4 (11%) | 10 (28%) | 10 (28%) |
| IgM $\lambda$ | 17 | 2 (5.5%) | 3 (11%) | 3 (11%) |
| Total | 53 | 6 (11%) | 13 (24%) | 13 (24%) |
| CD5$^-$ | | | | |
| IgM $\kappa$ | 19 | 5 (26%) | 0 (0%) | 0 (0%) |
| IgM $\lambda$ | 30 | 9 (30%) | 0 (0%) | 0 (0%) |
| Total | 49 | 14 (28%) | 0 (0%) | 0 (0%) |

In this study the expression of heavy chains from the $V_H$IV family was associated with antibodies that were polyreactive (MacKenzie, L.E., R.A. Mageed, and P.M. Lydyard, unpublished observations). However, $V_H$IV gene family usage was not seen to be a prerequisite for polyreactivity since heavy chains from other gene families were also expressed in polyreactive antibodies in both $CD5^+$ and $CD5^-$ cell lines.

The significant findings of the present study are: (*a*) The high frequency of $V_H$IV gene family expression observed in cell lines established from cord blood B cells, and (*b*) The selective expression of a subset of $V_H$IV genes, characterized by the expression of the R2.1A2 and 9G4 CRI, within $CD5^+$ B cells.

The $V_H$IV gene family is composed of a maximum of about 10 members, and the incidence of paraproteins derived from this gene family is low in Waldenstrom's macroglobulinemia and multiple myeloma (12). In contrast the frequency of $V_H$IV gene usage in fetal liver B cells has been estimated to be 21% (16), a frequency not dissimilar to the 30% observed for the cord blood derived B cell lines.

The high frequency of $V_H$IV gene use in fetal and neonatal life may reflect an evolutionary selective pressure for the early production of antibody specificities encoded by this gene family or programmed expression of $V_H$ genes (17).

It may be presumed that the early antibody repertoire would be generated using rearranged germline gene segments with minimal somatic mutation (18). It has been shown that such germline encoded specificities include recognition of self antigens and it has been suggested that a network of self antigen recognizing antibodies contributes to the further development of the immune repertoire. It may be significant, therefore, that numerous poly-reactive autoantibodies have been shown to use $V_H$IV gene segments. The data presented in this paper suggests that $V_H$IV gene use differs between $CD5^+$ and $CD5^-$ B cells in cord blood. It remains to be determined whether the CD5 antigen is a marker for a distinct lineage of B cells expressing a restricted repertoire of antibody specificities or is an activation marker such that the gene usage observed in early B cells reflects stimulation by a restricted number of self antigens.

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