

# Selective Expression of a $V_HIV$ 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  $V_HIV$  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  $V_HIV$  gene use (30%) in B cells from cord blood. Furthermore, two mutually exclusive CRI associated with distinct subgroups of the  $V_HIV$  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)<sup>1</sup> 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<sup>®</sup> (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.

<sup>1</sup> Abbreviation used in this paper: CRI, cross-reactive idiotypes.

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<sup>+</sup> and CD5<sup>-</sup> B cells were infected with EBV using supernatant from the B95-8 marmoset line, and cultured in the presence of 2 × 10<sup>4</sup> 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, κ, and λ isotypes. Cell lines positive for a single heavy and light chain isotype only were investigated for the expression of V<sub>H</sub>I (G6 and G8), V<sub>H</sub>III (B6 and D12), and V<sub>H</sub>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<sub>H</sub>III subgroup and a V<sub>H</sub>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<sub>H</sub>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<sup>+</sup> 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 Waldenström'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<sup>+</sup> 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 μ 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 μ, κ, and λ, (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 Titretrek 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 × 2 tables using the chi-squared test (χ<sup>2</sup>) 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<sub>H</sub> 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<sup>+</sup> population of B cells and 49 from the CD5<sup>-</sup> population. Thirty-six of the 53 CD5<sup>+</sup> cell lines expressed K light chain, while 17 expressed λ light chain, a κ/λ ratio of 2:1, roughly the ratio found in circulating Igs. For the CD5<sup>-</sup> cell lines a considerably lower κ/λ ratio of 1:1.6 was observed.

Previous studies demonstrated a relatively high frequency of V<sub>H</sub>III subgroup expression in B cells from patients with chronic lymphocytic leukaemia which express surface CD5 (14). Within our K-expressing CD5<sup>+</sup> lines 12/36 (30%) expressed the V<sub>H</sub>III subgroup of light chain (Table 1), a frequency similar to the expected frequency of this light chain

**Table 1.** V<sub>H</sub>III Subgroup Expression within Cell Lines Established from CD5<sup>+</sup> or CD5<sup>-</sup> B Lymphocytes and Association with 17-109 CRI Expression

Cell line origin	Lines expressing V <sub>H</sub> III		Lines expressing 17-109		
	Total	% of K lines	Total	% of V <sub>H</sub> III lines	% of K lines
CD5 <sup>+</sup>	12	30	8	67	23
CD5 <sup>-</sup>	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- $V_HIV$ CRI		
			LC1	R2.1A2	9G4
DIN	IgM- $V_HIV$ $V_{\kappa}III$	Rheumatoid factor	+	-	-
COR	IgM- $V_HIV$ $V_{\kappa}III$	Rheumatoid factor	+	-	-
STR	IgM- $V_HIV$ $V_{\kappa}III$	Unknown	+	-	-
CAL	IgM- $V_HIV$ $V_{\kappa}II$	Unknown	+	-	-
ODO	IgM- $V_HIV$ $V_{\kappa}III$	Cold agglutinin	-	+	+
HAW	IgM- $V_HIV$ $V_{\kappa}II$	Cold agglutinin	-	+	+
AB26	IgM- $V_HIV$ $V_{\lambda}$	Polyreactive	+	-	-
AB17	IgG3- $V_HIV$ $V_{\kappa}III$	Polyreactive	-	+	+
AB44	IgA- $V_HIV$ $V_{\lambda}I$	Polyreactive	-	+	+

Reactivity of monoclonal anti-CRI was determined in ELISA.  $V_H$  and  $V_L$  subgroup of proteins DIN, COR, STR, CAL, ODO, and HAW were assigned by immunoblot analysis with peptide induced reagents. Expression of  $V_{\kappa}III$  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  $V_{\kappa}III$  in the K-expressing  $CD5^-$  lines was similar (26%) (Table 1). However, the frequency of  $V_{\kappa}III$  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  $V_HIV$ -associated CRI. This is of particular interest because recent studies by Sanz et al. (12) have demonstrated a remarkably high frequency of  $V_HIV$  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 ( $\sim 10$ ) and the high degree of internal homology (at least 91.5% between the most distant members to a consensus  $V_HIV$  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  $V_HIV$  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  $V_HIV$  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  $V_HIV$  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  $V_HIV$  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  $V_{\kappa}III$  epitope C7 or 17-109 together with R2.1A2 and 9G4. This appears to be in contrast to the observed association of  $V_HIV$  heavy chains and  $V_{\kappa}III$  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
<b>CD5<sup>+</sup></b>				
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%)
<b>CD5<sup>-</sup></b>				
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<sub>H</sub>IV family was associated with antibodies that were polyreactive (MacKenzie, L.E., R.A. Mageed, and P.M. Lydyard, unpublished observations). However, V<sub>H</sub>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<sup>+</sup> and CD5<sup>-</sup> cell lines.

The significant findings of the present study are: (a) The high frequency of V<sub>H</sub>IV gene family expression observed in cell lines established from cord blood B cells, and (b) The selective expression of a subset of V<sub>H</sub>IV genes, characterized by the expression of the R2.1A2 and 9G4 CRI, within CD5<sup>+</sup> B cells.

The V<sub>H</sub>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 Waldenström's macroglobulinemia and multiple myeloma (12). In contrast the frequency of V<sub>H</sub>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<sub>H</sub>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<sub>H</sub> 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<sub>H</sub>IV gene segments. The data presented in this paper suggests that V<sub>H</sub>IV gene use differs between CD5<sup>+</sup> and CD5<sup>-</sup> 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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