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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_κ 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_κ 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%: $P < 0.05$). 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_κ, 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_κ 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 μg/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 μl 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_κ IIIb/V_H I) from the Wa cross-reactive idiotype family and He (V_κ 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_κ 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_κ 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_κ IIIb sub-subgroup associated idiotope.

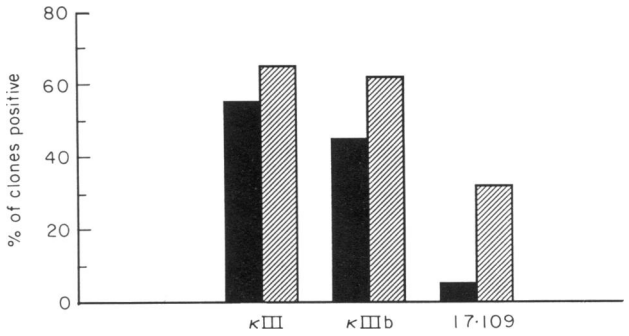

Fig. 1. More frequent expression of the V_κ IIIb sub-subgroup associated idiotope 17.109 by $CD5^+$ than $CD5^-$ kappa clones. $P < 0{\cdot}025$. V_κ III subgroup and V_κ IIIb sub-subgroup associated isotypic determinants were detected using an ELISA with MoAb C7 and C6 respectively. The V_κ IIIb sub-subgroup 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 μg/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 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 units at 492 nm compared with background values of less than 0·05. Monoclonal anti-μ, κ and λ light chains were clones AF6, 6e1 and C4 respectively (Oxoid-Unipath, Bedford, UK).

RESULTS

More frequent use of kappa light chains and V_κ IIIb sub-subgroup associated idiotope 17-109 by $CD5^+$ B cells

Of 86 clones derived from $CD5^+$ B cells, 54 used κ 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 κ clones studied, the frequency of the V_κ III sub-group and the V_κ IIIb sub-subgroup both $CD5^+$ and $CD5^-$ clones was similar (Fig. 1). In both groups the V_κ IIIb sub-subgroup represented >75% of the V_κ III family chains used, as detected using the MoAb C6. However, more of the $CD5^+$ clones (11 of 34) expressed the V_κ 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

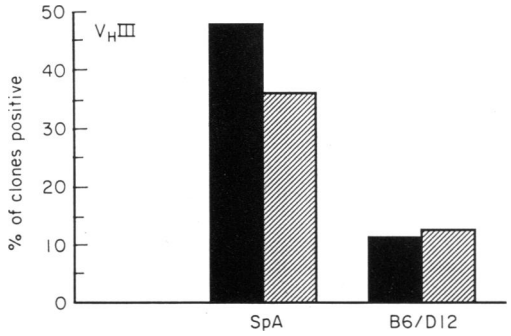

Fig. 2. Similar frequency of V_H I and V_H III associated CRI expressed by IgM from CD5⁺ and CD5⁻ clones. G8 (a V_H I associated CRI) and B6 and D12 (V_H III associated CRI) MoAb were used in ELISA as described in Materials and Methods. IgM from 45 CD5⁻ and 40 CD5⁺ clones was examined. IgM from 46 CD5⁻ 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 ⩾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⁻ κ clones expressed the idiotope G8. All four of these G8 positive clones were associated with kappa chains using V_κ III family genes and two were V_κ IIIb positive (data not shown). One λ 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⁺, κ and λ light chain expressing clones were also seen in both populations. The V_HIII 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 3λ 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⁺ κ light chain expressing clones which used V_κ IIIb together with the V_H I associated CRI, G8 was polyreactive, reacting with Fc, ssDNA and perinuclear antigen. The other κ clone did not react with any autoantigen in the panel tested. One of the G8 positive, CD5⁻ clones which expressed V_κ III was polyreactive whilst the other used V_κ IIIb subgroup of light chain and reacted with ssDNA and mitochondria. Only the CD5⁻ λ 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_HI 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 κ or λ chains (data not shown).

Expression of V_κ family sub-groups and associated idiotopes

The frequency of κ 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 IgMκ expressing clones which were auto and polyreactive to the panel of antigens tested, was higher than the λ light chain bearing clones in both CD5$^+$ and CD5$^-$ groups. However, this was only significant within the CD5$^+$ population where 34/54 κ clones compared with 10/32 λ 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_κ 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_κ 325 which is associated with the V_κ 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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