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Detection of Pre-Malignant B-1 Cells in NZB Mice with a Restricted CDR3/DFL16 Region

Fereidoun Mahboudi[*1], Ann. J. Feeney[2], Larry Arnold[3], Geoffrey Haughton[3], and Elizabeth Ravech[4]

[1]Biotechnology Department, Pasteur Institute of Iran, Tehran 13164, Iran; [2]Scripps Research Institute, La Jolla, CA.; [3]University of North Carolina, Chapel Hill, NC USA; [4]Department of Pathology and Laboratory Medicine, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, NJ 07103 USA

ABSTRACT

The relationship between the immunoglobulin (Ig) nucleotide sequence and the ability of a B cell to develop into a malignant cell was studied in a subset of B cells, B-1 cells. B-1 cells become malignant in chronic lymphocytic leukemia (CLL) and are responsible for the production of "natural autoantibodies". The autoimmune NZB mouse has been known as a human malignancy and CLL model, because of the age-dependent onset of clonally expanded hyperdiploid B-1 cells in these mice. The Ig heavy chain variable region in hyperdiploid B-1 clones from several NZB mice showed common characteristics in the CDR3 shared with fetal B cells: lack of N base insertions and presence of homology sequences at the V_H-D-J_H junctions that can be encoded by either of the two joined gene segments. Using a degenerative oligoprimer was shown no significant differences in expression of the restricted CDR3/DFL16 region in newborns or in the liver of either strain of mice as young adults. However, the expression of the restricted CDR3/DFL16 in the spleens of young adult NZB was remarkably elevated and showed significant differences from the expression in newborn NZB as well as from young adult and newborn BALB/c mice. It appears that malignant hyperdiploid B-1 cells are derived from fetal B cells. This technique can be used as a molecular marker to demonstrate a relative increase in the expression of this CDR3 in animals pre-destined to develop B-malignancies.

Keywords. pre-malignant B-1 cells, CDR3, junctional homology, quantitative PCR

INTRODUCTION

The insight gained through molecular analysis of immunoglobulin heavy chain variable (Igh) region has remarkably changed our understanding of growth and selection of B cells [1, 2]. Immunoglobulin genes undergo rearrangements which involve joining of V_{Hs}, D_{Hs}, and J_H through two successive somatic recombinations. The large number of V_{Hs}, D_{Hs} and J_H gene segments create initial diversity in Ig variable heavy chain. Moreover, the deletion of nucleotide sequences at the ends of coding segments and addition of non-germline sequences, N bases, at the junctional region creates more diversity [3-5].

The Ig heavy chain genes are rearranged during pro and pre-B- cell development [1]. The molecular analysis of the Ig variable region, particularly the junctional regions, can be helpful to determine the progenitor cells of different B cell subsets. Gu et al. found that the frequency of N base insertions in the V_H-D-J_H junctions of adult pre-B cells is significantly higher compared to neonatal pre-B cells. Likewise, they illustrated that the B-1 cells and classical B cells possess different kinetics of N base insertions during the course of ontogeny [6]. In addition, the frequency of N base insertions in fetal B cells compared to the adult B cells have been shown to be significantly different [7, 8]. Therefore, it seems that insertion of non-germline N bases insertions is a "developmentally regulated process" in precursors of murine B lymphocytes.

Another feature that differentiates fetal B cell from adult B cells is the presence of one to six nu-

*Corresponding author. Abbreviations: CLL, Chronic Lymphocytic Leukemia; CDR3, complementarity determining region 3; Igh, Immunoglobulin heavy chain ; V_H, Immunoglobulin heavy chain variable region gene; TdT, terminal deoxynucleotidyl transferase; RT-PCR, reverse transcription polymerase chain reaction; 2MON, two month old NZB; 2MOB, two month old BALB/c; 1DOB, one day old BALB/c; 1DON, one day old NZB; Hl-Fl: hybridoma of conventional B cells derived from the spleen of a 6 month old (NZB x DBA/2)Fl.

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cleotide homology sequences at the junctional regions that could be encoded by either of two adjacent gene segments [7-10]. These homology sequences could facilitate recombination at those sites [9, 11-13]. For most Ig V_H-D and D-J_H junctions, there are only 1-2 short stretches of homology, and therefore only 1-2 junctional sequences which are predominantly observed in fetal B cells [14]. Thus, fetal Ig heavy chain variable regions display limited junctional diversity, due to the lack of N regions and to homology directed recombination. Lack of N region is due to lack of terminal deoxynucleotidyl transferase (TdT) expression early in ontogeny [15, 16]. The clonal expansion of B-1 cells in old NZB mice has been proposed as an animal model for the human $CD5^+$ B cell malignancy, CLL [17]. By one year of age, NZB mice display clonal hyperplasia of B-1 cells which is long lived, slow growing, immunoregulatory, and possessing specific extra chromosomes [reviewed in 18]. These hyperdiploid B-1 cells are long lived to the point of virtual immortality. We have previously shown that NZB hyperdiploid cells can be passaged by i.v. injection into unirradiated (NZB x DBA/2) F1 recipients. In general, the passaged hyperdiploid cells are found mainly in the spleen, peripheral blood, and the peritoneal cavity of the recipient animal. An *in vitro* line of the hyperdiploid B-1 line, P-13L, has been established in the laboratory. Immunoglobulin VH genes of hyperdiploid B-1 cells derived from old NZB mice, the *in vitro* line, and recipients of passaged hyperdiploid B-1 cells showed a lack of somatic mutations [19]. In the present report, the malignant B-1 clones of NZB mice were found to possess CDR3 regions with fetal characteristics, including sequence homology at the junction sites and no N base insertions [20-22]. These characterisitcs were exploited in an attempt to identify pre-malignant B cells in NZB mice with similar CDR3 regions [23-27].

By generating a CDR3 specific oligonucleotide, Yamada et al. were able to detect the DNA sequences encoding the CDR3 of Igh-rearrangement in acute lymphocytic leukemia (ALL). They were able to detect residual leukemic disease in most patients with malignancies of the B-cell lineage [20]. In the present studies we identified CDR3 regions with fetal characteristics in the majority of NZB hyperdiploid B-1 clones. Employment of a CDR3/DFL16 oligonucleotide as a primer in a PCR reaction allowed for detection of subpopulation of cells pre-destined to become clonally expanded in young NZB mice. V_H-D-J_H sequence diversity at the junctions of Ig variable region differ between fetal and adult B cells in terms of lack of N base insertions and presence of homology at juctional overlaps.

MATERIALS AND METHODS

Mice: NZB, DBA/2, and BALB/c mice were obtained from Jackson Labs (Bar Harbor, ME) and (NZB × DBA/2) Fl animals were bred in our animal facility. Spleen cells were transfused into (NZB x DBA/2) Fl animals by i.v. injection as described [17]. Animals were sacrificed by cervical dislocation under light ether anesthesia. For analysis of fetal sequences published in this manuscript, fetal liver DNA was isolated from 18 day old embryos of ($B10.H2^aH4^bIgh^b$ x $B10.H2^aH4^bIgh^a$) Fl mice. Additional analyses of fetal sequences were obtained from published reports and unpublished analysis of BALB/c liver DNA from Feeney and her group.

Primers for PCR reaction: Primers for RT-PCR of immunoglobulin RNA were Ci.t (downstream), CDR3/DFL16 (downstream) and V_H1BACK (upstream) primers synthesized by Operon Technologies, Inc. (Alameda, CA) and used without further purification. The Cμ primer was [5'-d(GCAGGAGACGAGGGGGAA)]. The complementary sequence primer to the 5' half of the CDR3/DFL16 region found as a common element in the expressed Ig of several hyperdiploid NZB B1 clones regardless of their V_H and J_H regions was 5' AGTAGCTACTACCGTAGTAAKM 3' (K=G or T; M= A or C). Based on the %GC content in this primer the Tm was 49° C with no primer-dimer formation which was determined by the computer program Amplifyer, (Amplifyer, for analyzing PCR experiment, University of Wisconsin, Madison, WI). The V_H1 primer is a consensus sequence to all known murine immunoglobulin framework 1 regions. The sequence is 5'-d(AGGTSMARCTGC AGSAGTCWGG) (R=A or G; W=A or T; S=G or C). A low annealing temperature (37° C) was used during PCR amplification to compensate for the degeneracy of this primer. For amplification of V11 sequences in fetal DNA, primers employed were

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V11 (upstream) and J_H (downstream). The V11 primer was located in the CDR1 and was 5' ACT GAT TAC TAC ATG AGC TGG GTC C and the J_H primer was four separate JH primers which were 24mers and found at the 3' end of each J_H.

cDNA Synthesis, PCR Amplification and Sequencing: Total RNA was prepared by the GTC-acid phenol method. Reverse transcription and PCR protocols were based on and all reagents (except specific primers) were included in the Perkin Elmer Cetus GeneAmp RNA PCR kit (Perkin Elmer Cetus Corp., Norwalk, CT) [28-31]. The sample (either RNA or DNA) was then subjected to PCR employing in an Ericomp thermal cycler (Ericomp Inc, San Diego, CA). PCR sample quality was examined on a 3% NuSieve agarose gel (FMC Bioproducts, Rockland, ME). The PCR product was cloned into pCR1000 plasmid (In Vitrogen, San Diego, CA) and sequenced by the Sanger dideoxy method. The NZB sequences were analyzed on an Applied Biosystems DNA Sequencer (Applied Biosys tems, Foster City, CA).

Quantitative PCR: The CDR3 /DFL16 primer was endlabelled with ATP (y-^{32}P) according to the manufacturer's instructions (BRL) prior to use in RT reactions. To be certain that all the primer sequences are labeled, the ratio of the oligo primer to the ATP (y-32P) was reduced to the minimum possible levels (8 pmol of the oligo primer and 14 pi of the ATP (y-32P). The PCR product was ethanol precipitated and run on 2% agarose gel. The proper bands were cut and cpm was obtained by scintillation counting. The % of relative expression of the specific CDR3 was calculated as follows:

$$\%\ \text{relative expression of CDR3} = \frac{CPM\ of\ CDR3\ bands}{CPM\ of\ C\mu\ bands} \times 100$$

RESULTS

Identification and immunoglobulin sequence analysis of NZB hyperdiploid B-1 clones: Five hyperdiploid B-1 clones from one year old NZB mice have been sequenced. Three of the Igh sequences which employed the DFL16 gene family were analyzed in more detail for the presence of a characteristic CDR3 region. The V_H-D and D-J_H junctions of sequence are shown in Figure lA and 1B. In each of the V_H-D junctions, 2 nucleotide sequence homologies which could be encoded by either the V_H or D genes were observed (Fig. 1A).

In contrast to V_H.D junctions, the D-J_H junctional sequence had three to six nucleotide overlaps (Fig.1B). In addition, unlike the V_H- D junctional overlap sequences, the D-J junctional overlap sequences are similar in several N2,3 B-1 malignant clones. Three different D-J_H junctional sequences all have TAC in common.

Clones	Positions	Overlapping Sequence
NZB-1	S107 V11	AGA GAT ATA
	Junction	AGA GAT TAC TAC GG
	DF116.1	*A* AT T TAT TAC TAC GG
NZB-2	J558	GCA AGA *TCT T*
	Junction	GCA AGA TCT TAC TAC
	DF1 16.1	*A* ATT TAT TAC TAC
NZB-3	V_H12	GGA GAT
	Junction	GGA GAT TAC TAC
	DF116.2	CAT TAC TAC

Fig. 1A. Igh V_H-D junctional sequences of NZB B-1 clones derived from three individual NZB mice. Bolded nucleotides represent homology directed recombination sites which can be derived from either gene. Italic bases represent palindromic bases. The top line in each group is the sequence of the 3' end of the reported VH germline gene. The middle line in each group is the V_H-D junctional sequence observed in the NZB B-1 clone reported sequence of the 5' end of the germline D gene. The bottom line is the reported sequence 5' end of the germline D gene.

Clones	Positions	Overlapping Sequence
NZB-1	DFL16	AGC **TAC** *GT*
	Junction	AGC **TAC** TAT GCT
	J_H4	*T*AT **TAC** TAT GCT
NZB-2	DFL16	AGC **TAC** GT
	Junction	AGC **TAC** TGG TAC
	J_H1	*AGC* **TAC** TGG *GT*
NZB-3	DFL16	TAC GGC **TAC** *GT*
	Junction	GGC **TAC** TGG TAC
	J_H1	*AGC***TAC** TGG TAC

Fig. 1B. Igh D-J_H junctional sequences of NZB B-1 clones derived from three individual NZB mice. Bolded nucleotides represent homology directed recombination sites which can be derived from either gene. Italic bases represent palindromic bases. The top line in each group is the sequence of the 5' end of the reported D germline gene. The middle line in each group is the D-J_H junctional sequence observed in the NZB B-1 clone. The bottom line is the reported sequence of the 5' end of the germline JH gene.

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Comparison of V_H-D and D-J junctional region in NZB B-1 malignancies and fetal and neonatal B cells: A comparison of the sequence at the sites of junctions in the adult malignant B-1 clones with sequences obtained from fetal B cells was performed. We compared sequence data obtained from 18 old day (B10.H2^{a}H4^{b}Igh^b × B10.H2^{a}H4^{b}Igh^a) F 1 embryos in which using an upstream primer for V11 gene family with old NZB. In the Fl fetus, 88% (16/18) of the reproductive rearrangements of both Igha and Ighb allotype which employ V11 had sequence homology at the site of junctions (unpublished data). Nearly all the productive rearrangements in the 18 day old F 1 embryo are similar to the NZB B-1 malignancies in that they employ regions of homology at the sites of junction in the heavy chain CDR3. The Igh heavy chain CDR3 sequence from NZB B-1 malignant cells employing the V11 possessed AT overlap sequences. Figure 2 shows the comparison of NZB B-1 V11 and DFL16.1 overlap sequences with overlap sequences from 18 day old F 1 embryos which employed the V11 and the DFL16.1 genes. In addition, analysis of the productive rearrangements in 18 day old Fl embryos which employed VII demonstrated that 17/18 (both Igha and Ighb sequences) had homology sequences at the D-JH junction site. The similarity between Igh sequence of NZB B-1 malignant clones and fetal sequences suggest that the NZB B-1 malignant clones arise during fetal development.

Sources	Position	Sequence
ZB-1N	V11	AGA **GAT** *ATA*
	Junction	AGA **GAT** TAC TAC
	DFL16.1	AAT **TAT** TAC TAC
Fl Embryo (18 day old)	V11	AGA **GAT** TAC TAC
	Junction	AGA **GAT** TAC TAC
	DFL16.1	TT **TAT** TAC TAC

Fig. 2. Comparison of Igh V_H-D junctional sequences from a malignant NZB B-1 clone which employed V11 and a representative junctional sequence from 18 day old fetal liver employing the Igh^a germline V11 sequences. Bolded nucleotides represent homology directed recombination sites which can be derived from either gene. Italic bases represent palindromic bases. The top line in each group is the sequence of the 3' end of the V 11 germline gene. The middle line is the V_H-D junctional sequence observed either in the NZB B-1 clone or fetal B cells. The bottom line is the reported sequence for the 5' end of the germline D gene.

Identification of a characteristic CDR3/DFL16 region in hyperdiploid NZB B-1 clones and the development of a quantitative PCR reaction: Before determining the relative expression of this characteristic CDR3/DFL16 region in young NZB mice prior to the development of malignant hyperdiploid B-1 clones compared to normal BALB/c, the degenerative oligoprimer complementary to the restricted CDR3/DFL16 was used as the downstream primer in control PCR reactions. Using radiolabelled primers, either the restricted CDR3/DFL16 primer or the CIA primer as the downstream primer, exponential accumulation of PCR products of different concentrations of template RNA from the positive control NZB-3, which had previously been sequenced and known to possess the restricted CDR3/DFL16, is shown in Figure 3. The specificity of the restricted CDR3/DFL16 oligoprimer was confirmed by using different concentration of RNA from a negative control source of RNA, H1-F1 which is an IgM producing hybridoma which had previously been sequenced and known not to employ the restricted CDR3 (Figure 4). This control source of RNA had N base insertions and had the following CDR3 (5' AGATGGGGCGGGACCTCTTAC3'). Both the positive (NZB-3) and the negative (H1-F1) sources of mRNA were able to be primed in the PCR reaction by the Cμ downstream primer. In contrast, only the source of hyperdiploid NZB B-1 clones (NZB-3) was able to produce a PCR product when primed with the CDR3/DFL16 downstream primer.

Prior to the application of this technique into a heterogeneous population such as spleen or liver to reveal the relative expression of the restricted CDR3/DFL16 compared to expression of Cμ, a mixture of different concentration of positive and negative control template RNA were used in a RT-PCR reaction (Figure 5). In this experiment a mixture of different concentration of Hl-F1 and NZB-3 RNA (total 1 μg) was reverse transcribed, cDNA was amplified for 18 cycles using a degenerative V_H upstream and either Cμ or CDR3/DFL16 downstream primer. It can be seen that the total amount of PCR product using Cμ primer are almost the same, whereas the amount of PCR product using the restricted CDR3/DFL16 declined as the amount of the positive control was reduced. The amount of RNA from a negative source did not affect the yield of PCR product and did not inhibit the specific reaction in a heterogeneous mixture. Therefore, the

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chance of non-specific binding is very low and the primer will be very specific for Ig sequences that employ this restricted CDR3/ DFL16.

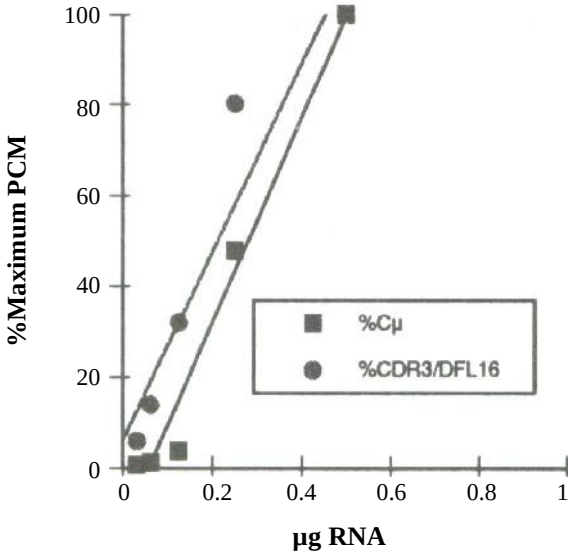

Fig. 3. Quantitative PCR of NZB-3 (positive control) using a degenerative V_H upstream primer and a radiolabeled downstream primer either CDR3/DFL16 or Cμ primer. Closed squares represent % of PCR products using a Cμ primer which will PCR all IgM mRNA. Closed circles represent % of PCR products using a degenerative CDR3/DFL16 primer. % maximum cpm was calculated as the cpm ratio of PCR product of each particular concentration of RNA to the cpm of PCR products generated from the highest concentration of RNA used multiplied by 100.

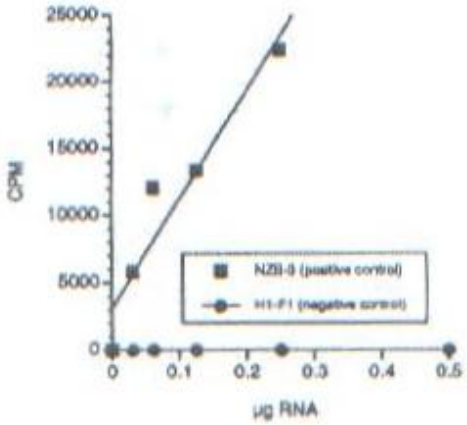

Fig. 4. Priming ability of the restricted degenerative CDR3/DFL16 primer as the downstream primer in a PCR reaction. The CDR3 primer was end-labeled prior to use. PCR products were electrophoresed, the proper bands were cut and counted with a scintillation counter. Closed circles represent cpm of PCR products generated by RTPCR of RNA from the negative control H1-F1 which is a hybridoma derived from a conventional F1 B cell. Closed squares represent cpm of PCR products generated by RT-PCR of RNA from the positive control NZB-3.

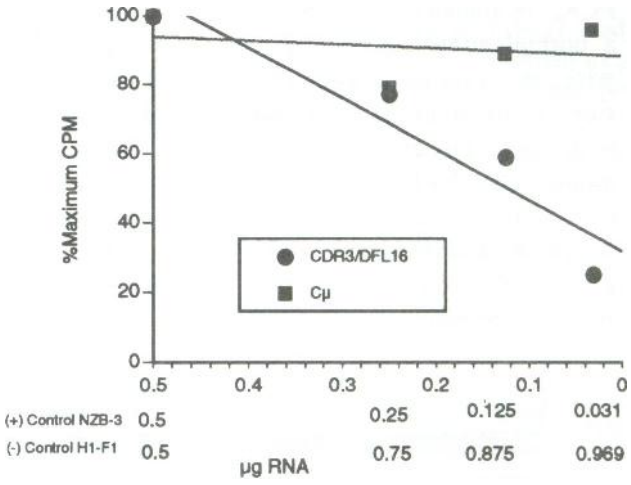

Fig. 5. Quantitative of PCR products from a mixture of positive and negative control RNA. NZB-3 RNA derived from NZB hyperdiploid B-1 cells which had an Igh sequences containing a CDR3 which could be primed by the CDR3/DFL16 primer. HI-Fl is a hybridoma derived from conventional B cells which did not possess the CDR3 sequence which could be primed by the CDR3/DFL16 primer. The total RNA in the PCR reaction was lug. The amount of RNA derived from each sample is indicated. The closed circles represent the % of PCR product using the Cu primer. The closed squares represent % of PCR product using the restricted CDR3/DFL16 primer. The curves were plotted based on linear regression. % maximum cpm was generated as:

$$\frac{cpm\ of\ PCR\ products\ from\ each\ samole}{cpm\ of\ the\ PCR\ product\ of\ the\ highest\ concentration} \times 100$$

Relative expression of the restricted CDR3/DFL16 region in liver and spleen of NZB and BALB/c: To determine if young NZB mice have an increased percentage (relative to normal mice) of B cells which express the characteristic CDR3/DFL16 found in both fetal B cells and many NZB B-1 malignancies, quantitative PCR using either the Cμ primer or the CDR3/DFL16 downstream primer was performed. Figure 6 shows the PCR product levels in liver and spleen of one day and two month old NZB and BALB/c mice using radiolabelled Cμ, or restricted CDR3/ DFL16 primers. Two months was chosen as a time point since both strains studied have a mature immune response and the NZB strain begins to develop oligoclonal expansion of B-1 cells yet has not developed detectable

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malignant hyperdiploid B-1 clones. The relative amount of cDNA expressing the restricted CDR3/DFL16 region, as determined by quantitative PCR, in spleen of BALB/c, which is considered as a normal strain of mice, declined with age. Likewise, the relative amount of CDR3/DFL16 expression declined in liver of both BALB/c and NZB with age. The analysis of the relative increase in 2 month old NZB B cells of the characteristic CDR3/DFL16 was detected with a PCR primer which is restricted to the 5' portion of CDR3 regions which employ only the DFL16 D_H gene with sequence homology and no N bases.

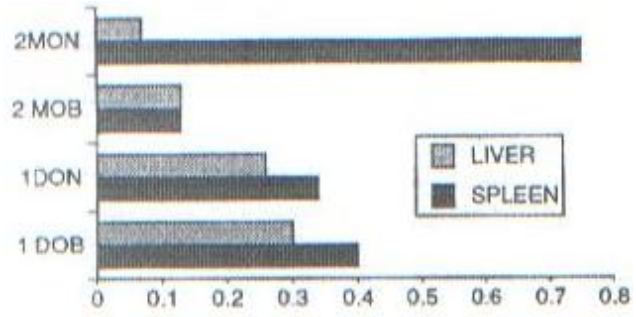

Fig. 6. Relative expression of the restricted CDR3 in NZB and BALB/c mice. 0.5 p.g RNA of spleen and liver was extracted, reverse transcribed and amplified for 18 cycles using an end-labeled downstream primer (either CDR3 or CO and a V_H degenerative primer as the upstream primer. Numbers represent

$$\% \, of \, \frac{cpm \, of \, CDR3 \, bands}{cpm \, of \, C\mu \, bands}$$

DISCUSSION

Fundamental differences at the molecular level between B-cell repertoires in fetal and adult stages have been illustrated by several investigators [6, 7, 14]. Previous reports have shown that B-1 cells frequently employ unmutated V_H genes [32]. As well, the immunoglobulin heavy chain gene from NZB hyperdiploid B-1 clones and unirradiated recipients of hyperdiploid B-1 clones employed unmutated germline genes. Most of B-1 cells employ DFL16 D_H gene family without N base substitutions [24]. The number of N sequences increases with age in pre B cells and B cells from normal mice [9] due to the low level of (TdT) expression at early age [15-17]. To date, there is no evidence of N sequences in hyperdiploid B-1 cells from one year old NZB, therefore cells with the potential to become hyperdiploid B-1 may also be derived early in ontogeny. In this study it was shown that four hyperdiploid B-1 clones were similar in the CDR3 region employed. Possession of a restricted CDR3/DFL16 as a common element in NZB hyperdiploid B-1 cells, makes the CDR3 a possible element which may play an important role in clonal expansion. Since the CDR3 comprises sequences from V_H, D and J_H gene segment, the CDR3 region in each immunoglobulin variable heavy chain is unique in each particular clone. The presence of the restricted CDR3 can be an identification tool for members of the same B-cell clone [20].

Sequence analysis of 3 NZB B-1 clones showed that Igh sequences possess one to six nucleotide homology sequences at the V_H-D-J_H junctions. The overlap sequences at the V_H-D junctions were shorter (two bases) than those at the D-J_H junctions (three to six). The longest homology was six nucleotides found between DFL16 and J_H1. Homology sequences at the V_H-D junctions were short and near the two coding ends to be joined [27]. The V11-DFL16.1 junction, the DFL16.1-41 junction, and DFL16.1-J_H4 junctions are identical to the ones shown to be predominant in neonatal Igh [14]. It appears therefore that hyperdiploid B-1 cells may originate from fetal B cells rather than adult B cell progenitors in which substantial N base addition and lack of homology sequences is common Although sequence homology is observed in fetal and newborn Igh sequences, it is unclear the role homology overlaps play in directing V_H-D-J_H recombination [33, 34].

We have used the restricted CDR3/DFL16 that identified a common region in several independent malignant hyperdiploid B-1 cells to detect premalignant clones. A degenerative oligoprimer complementary to the restricted CDR3/DFL16 was generated and used as a molecular marker to demonstrate the relative expression of the restricted CDR3 region in spleen and liver from newborn and young adult NZB and BALB/c. It was shown that the relative expression of the restricted CDR3 region increased dramatically in the spleen from NZB mice while decreased in the spleen from BALB/c with age. This primer also will prime those fetal B cells and B-1 cells utilizing the restricted CDR3/DFL16 region. It is possible to speculate that this restricted CDR3 region may be important in the clonal expansion of these cells in NZB mice.

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Multiple factors may be involved in the development of malignant hyperdiploid B-1 clones. It appears that NZB mice possess all the requirements for clonal expansion. This restricted CDR3 primer allows us to estimate indirectly the relative number of cells that are malignant or have the potential to become malignant B-1 cells. It appears that B-1 malignant clones seen at one year of age in NZB mice arise from premalignant cells that arise early in life. This strain of mice has been known as a murine model for autoimmune diseases [35-38]. Other investigators have described indolent clones of B-1 cells in aged normal mice [39]. One study concluded that B-1 lymphomas arising late in life in (C57BL/10.H H-2^aH-4^b) mice arose from premalignant clones of cells that had become committed to neoplasia early in development [40]. Other studies also suggest that the differentiation pathway which results in the development of B-1 cells is already established during the pro-B cell stage [41].

In summary, hyperdiploid malignant B-1 cells may originate from fetal B cells based on the molecular characteristics of the immunoglobulin heavy chain variable region shared with fetal B cells [9, 14]. The restricted CDR3 oligoprimer described here can be used for detection of hyperdiploid malignant B-1 cells or those fetal and newborn B cells that have the potential to become malignant. The presence of fetal nucleotide sequences within the CDR3 of pre-malignant B-1 cells may be useful for both early diagnosis and treatment of CLL.

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