The transition of pre-BI to pre-BII cells is dependent on the V_H structure of the μ /surrogate L chain receptor

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We have demonstrated previously that the majority (>90%) of V_H12 B cells are absent from the adult peripheral repertoire, and that most that remain have a V_HCDR3 sequence of 10 amino acids with a Gly in the fourth position at the D–J junction (designated 10/G4). We report here that most V_H12-expressing pre-B cells are lost during the transition from the pre-BI to the pre-BII cell stage in normal mice, and that pre-BII cell productive (P) rearrangements are enriched in 10/G4 CDR3. This coincides with the initial expression of H chain and the generation of the μ /surrogate L chain (SL) receptor. In contrast, there is no enrichment for 10/G4 CDR3 in μ MT mice, and the frequency of P rearrangements is as expected from a random rearrangement mechanism, ruling out a biased rearrangement mechanism unique to V_H12. We have also demonstrated that non-10/G4 μ chains can associate with SL and be expressed on the cell surface, suggesting that they are available on the cell surface for selection. Thus, transition of pre-BI to pre-BII cells is dependent on the structure of the V_H domain.

Keywords: positive selection/pre-B cells/V_H12 rearrangements

Introduction

B cell precursors undergo a series of Ig gene rearrangements and selection in the bone marrow (BM). Rearrangement of the heavy chain gene locus occurs first, beginning with a D to J_H rearrangement to generate pre-BI cells, and then a V_H to DJ_H rearrangement to generate cytoplasmic μ^+ pre-BII cells. Pre-BII cells undergo light chain gene rearrangement to generate surface IgM⁺ B cells. At the pre-BII cell stage, a complex consisting of the μ heavy chain and surrogate light chain (SL), composed of λ 5 and VpreB proteins, is expressed on the cell surface (Rolink *et al.*, 1994; Winkler *et al.*, 1995). Although the exact function of the μ /SL complex is not known, 'knock-out' of either the λ 5 or μ chain gene leads to inability to generate pre-BII cells (Kitamura *et al.*, 1991, 1992; Ehlich *et al.*, 1993), suggesting that the μ /SL complex is required for normal pre-B cell development. Presumably, ligand binding by this complex generates a positive signal essential for the transition from pre-BI to pre-BII. This is consistent with the finding that treatment of pre-B cell

lines with anti- μ or anti- λ 5 can trigger a signal transduction cascade (Misener *et al.*, 1991). The nature of the ligand remains unclear, although it has been speculated that it may be a molecule on BM stromal cells.

The peripheral repertoire of B cells is affected by negative (Nemazee and Buerki, 1989; Hartley *et al.*, 1993) and probably positive selection (Gu *et al.*, 1991a). As B cell development involves distinct stages, the question of when selection occurs has received much attention. Many studies have indicated that IgM⁺ immature B cells are subject to negative selection that results in the elimination of self-reactive B cells (Nemazee and Buerki, 1989; Hartley *et al.*, 1993). In addition, studies of V_H gene usage indicate that a V_H-dependent selection mechanism operates at the IgM⁺ stage. For example, the ratio of V_HJ558 to V_HJ7183 family gene use, and the expression frequency of individual members of the V_HJ558 family, are different between BM pre-B and peripheral B cells (Freitas *et al.*, 1990; Malynn *et al.*, 1990; Gu *et al.*, 1991a). On the other hand, others (Decker *et al.*, 1991a,b; Huetz, 1993) have analyzed the frequency of productive (P) relative to non-productive (NP) rearrangements in pre-BII cells and found that it is higher for V_H36–60, but lower for V_H81X, than expected in the absence of selection, raising the possibility that V_H-dependent selection can occur at the pre-BII cell stage.

Our recent analysis of V_H12 rearrangements suggests that V_H12-expressing B cells also go through a selection process in adult BM and that CDR3 is involved in this selection (Clarke and McCray, 1993; Ye *et al.*, 1995). An estimated >90% of the expected V_H12-expressing B cells are missing in the adult spleen and peritoneum and ~90% of the remaining V_H12 B cells have a 10/G4 V_HCDR3. To explain these data, we have proposed selective survival of V_H12-expressing B cells during differentiation based on the CDR3 sequence. Thus, V_H12-expressing B cells with a 10/G4 CDR3 are positively selected by interaction with ligand and continue their differentiation, while the majority of V_H12-expressing B cells with other V_HCDR3 sequences (non-10/G4 CDR3) are not positively selected due to failure to bind ligand, and consequently undergo programmed cell death (PCD) (Wyllie, 1980; Cohen and Duke, 1992).

To determine when selection of V_H12-expressing B cells occurs, we have now examined the V_H12 rearrangements in the BM of normal mice as well as of μ chain 'knock-out' (μ MT) mice. We find that the majority of V_H12 P rearrangements are missing in the pre-BII cell repertoire in normal mice, and that these cells are enriched in 10/G4 V_HCDR3. However, P rearrangements are present at the expected frequency in μ MT mice where μ chain-based selection of pre-BII cells cannot occur. We also find that μ chains with a non-10/G4 CDR3 can associate with SL and are expressed on the cell surface. These results indicate

Table I. P/NP ratios of V_H12 rearrangements in BM of normal mice

P/NP ratio	Unsorted BM		BM B220 ⁺ /IgM ⁺	BM B220 ⁺ /IgM ⁻
	J _H 1	J _H 4	J _H 1	J _H 1
10/G4				
Expected	≤0.1	≤0.1	≤0.1	≤0.1
Observed	0.33 (6/18)	0 (0/22)	0.24 (4/17)	0.05 (2/38)
Non-10/G4				
Expected	≥2.1	≥2.1	≥2.2	≥2.0
Observed	0.22 (4/18)	0.23 (5/22)	0.18 (3/17)	0.11 (4/38)

All data are taken from Figures 1 and 2. Only those rearrangements amplified from genomic DNA are used for calculation of P/NP. The 10/G4 P/NP are the ratios of the total number of P rearrangements with the 10/G4 CDR3 to the total number of NP rearrangements, and the non-10/G4 P/NP are the ratios of the total number of P rearrangements other than those with a 10/G4 CDR3 to the total number of NP rearrangements. The number of P rearrangements (numerator) and NP rearrangements (denominator) is indicated in parentheses.

that normal development of V_H12-expressing pre-BII cells is dependent on the CDR3 structure of the μ chain. We suggest that expression of a μ /SL receptor is not by itself sufficient for normal development of pre-BII cells and that binding of ligand by this receptor involves the V_H domain.

Results

To determine the differentiative stage at which V_H12-expressing B cells are eliminated, we have analyzed V_H12 rearrangements from the BM of normal mice and μ MT mice. Rearrangements of V_H12–D–J_H1 or J_H4 were amplified by PCR and cloned into the TA cloning vector, and the resulting clones were chosen randomly for sequencing. Due to the tremendous diversity of V–D–J rearrangements in adult BM, the chance of identifying independently generated sequences that are identical is very small (Decker *et al.*, 1991b) and, therefore, sequences with an identical CDR3 are probably the result of *in vivo* clonal expansion or PCR amplification. To avoid the introduction of PCR-generated error, the repeats are counted only once, although by doing so the degree of clonal expansion may be somewhat underestimated. For this analysis, we have considered 10/G4 and non-10/G4 sequences separately because they are subject to different selective pressures (Ye *et al.*, 1995).

The majority of P V_H12 rearrangements are absent from the pre-BII cell repertoire in normal mice

The ratio of the total number of P rearrangements to the total number of NP rearrangements (P/NP) can be used to measure the representation of cells expressing a particular V_H gene. The P/NP for any given V_H gene in the absence of selection or bias in rearrangement is expected to be ~2.3 among μ^+ B cells, since all have a P rearrangement (Alt *et al.*, 1984; Loffert *et al.*, 1994). However, this ratio will be smaller for any population in which not all cells are μ^+ . For example, only 90% of the B220⁺/surface μ^- population in the BM are cytoplasmic μ^+ (Karasuyama *et al.*, 1994). As the P/NP for BM B220⁺/cytoplasmic μ^- cells is 0.5 (Chukwuocha *et al.*, 1995), the expected P/NP for the BM B220⁺/surface μ^- population used in this study should therefore be 2.1 (90% with a ratio of 2.3 and 10% with a ratio of 0.5). For unsorted BM cells in which B lineage cells (B220⁺) include ~50% B220⁺/

surface μ^+ and 50% B220⁺/surface μ^- cells (Arnold *et al.*, 1994), the expected P/NP should be 2.2.

A P/NP less than expected would be indicative of selection against cells with P rearrangements, and a ratio greater than expected would indicate clonal expansion of cells carrying P rearrangements (Decker *et al.*, 1991a,b; Carlsson *et al.*, 1992). It should be noted that for consideration of P rearrangements of only a single CDR3 sequence motif, the expected P/NP would be much less than the values given above, and would depend on the frequency with which this motif occurs by chance. Since not a single 10/G4 CDR3 is observed in 22 P rearrangements in μ MT mice (see below), the frequency of 10/G4 among P rearrangements is ≤1/23. Therefore, the expected 10/G4 P/NP (ratio of P rearrangements with a 10/G4 CDR3 to the total number of NP rearrangements) is likely to be ≤0.1 (2.3/23), and the expected non-10/G4 P/NP (ratio of P rearrangements that do not have a 10/G4 CDR3 to the total number of NP rearrangements) is likely to be ≥2.1 for unsorted BM cells, 2.2 for BM B220⁺/surface μ^+ cells and 2.0 for BM B220⁺/surface μ^- cells.

We initially examined V_H12 rearrangements to J_H1 and J_H4 gene segments in unsorted BM cells of normal mice. As shown in Figure 1 and Table I, the non-10/G4 P/NPs are nearly 10-fold lower than expected in both cases, indicating that the loss of V_H12-expressing B cells occurs in the BM. To determine whether this loss occurred at the pre-B or B cell stage, we analyzed V_H12–J_H1 rearrangements in FACS-sorted BM B cells (B220⁺/IgM⁺) and pre-B cells (B220⁺/IgM⁻). To obtain a larger sample size of pre-B cell P rearrangements, V_H12–J_H1 rearrangements were also amplified from pre-B cell cDNA.

As shown in Figure 2 and Table I, the non-10/G4 P/NPs for sorted B and pre-B cells are 12- to 18-fold less than expected, indicating a loss of ~95% of V_H12-expressing pre-BII cells with a non-10/G4 V_HCDR3. On the other hand, the 10/G4 P/NP ranges from 0.05 to 0.24, not lower than expected among unsorted BM cells or either of the sorted B lineage populations, suggesting that there has been no loss of V_H12-expressing pre-BII cells with a 10/G4 V_HCDR3. In fact, the 10/G4 P/NP among IgM⁺ B cells and unsorted BM cells is actually somewhat higher than expected. This may result from recirculating peripheral IgM⁺ B cells which contain clonally expanded PtC-specific B cells with a 10/G4 V_HCDR3 (Pennel *et al.*, 1989, 1992; Arnold *et al.*, 1994; Ye *et al.*, 1995) or clonal expansion of these immature cells in the BM.

Clone	V _H	P _V	N	P _D	D	P _D	N	P _J	J _H	D gene	a.a. seq

V _H 12-J _H 1											
Productive											
3	GGA	GAC			TAT GAT GGT				TAC TGG TAC TTC GAT GTC	D9	DYDGYWYFDV
10	GGA	GAC AG	C CAC		GGC AGT			AG C	TAC TGG TAC TTC GAT GTC	F2	DSHGSSYWFYDV
13	GGA	GAC AG			T GAT GGT				TAC TGG TAC TTC GAT GTC	D9	DSDGWYFVDV
15	GGA	GAC A	AA		GAT GGT				TAC TGG TAC TTC GAT GTC	D9	DKDGWYFVDV
19	GGA	GAC AG			C TCA G			G C	TAC TGG TAC TTC GAT GTC	?	DSSGYWYFVDV
30	GGA	G	CC CC		T TAC GAC	GT	C A		AC TGG TAC TTC GAT GTC	D2,3,4,6	VPYDNNWYFVDV
32	GGA	GAC			CTA ACT GG			C	TAC TGG TAC TTC GAT GTC	Q	DLTGWYFVDV
36	GGA	GAC AGA			TAT GGT				TAC TGG TAC TTC GAT GTC	D1,3-8	DRYGYWYFVDV
46	GGA	GTC	CG		T TAC TAC GGT AGT AGC TAC		CGG G		AC TGG TAC TTC GAT GTC	F1	VRRYGSYRDWYFVDV
47	GGA	GAC AG	C C		CC TAC TAT AGT AAC		CCG		TAC TGG TAC TTC GAT GTC	DX	DSPYYSNFWYFVDV
Non-productive											
4	GGA	GAC			CTA TGG TAA CTA C		G		GG TAC TTC GAT GTC	D1,5,7,8	
5	GGA	GAC AG			T CTA TGA TGG TTA CTA C		CA		TAC TGG TAC TTC GAT GTC	D9	
6	GGA	GAC	CCC		TTA CTA CGG TAG TAG C		CT TAG		GG TAC TTC GAT GTC	F1	
9	GGA	GAC	TCA		TAT TAC TAC GGT		CCC		AC TGG TAC TTC GAT GTC	F1	
11	GGA	GAC AG	C A		TA TGA CTA CG		G AGG		TGG TAC TTC GAT GTC	D2	
12	GGA	GAC			CT				TGG TAC TTC GAT GTC	?	
14	GGA	GAC A	AG AG		T ATT ACT ACG GTA				G TAC TTC GAT GTC	F1	
18	GGA	GAC AGA T	AA G		AT TAC TAC GGT TA		C TAC TC		AC TGG TAC TTC GAT GTC	F1	
20	GGA	GA		A	TCT ACT ATG ATT ACG		GGC CTC		G TAC TTC GAT GTC	D2	
21	GGA	GAC			TGG CCG AGG				GG TAC TTC GAT GTC	?	
24	GGA	GAC	CT		T ACT ATA GT				TAC TGG TAC TTC GAT GTC	DX	
33	GGA	GAC A	T		T CTA CTA TGA TTA CG				C TGG TAC TTC GAT GTC	D2	
35	GGA	GAC			TAG AGA				TGG TAC TTC GAT GTC	?	
37	GGA	GAC	TC		T TAT TAC TC		T AG		GG TAC TTC GAT GTC	F1	
38	GGA	GAC AGA	G		TA CTA CGG TAG TAG CT		A AG		GG TAC TTC GAT GTC	F1	
42	GGA	GAC	CCC CTC TC		A TTA CTA CGG TAG TAG		G		TAC TGG TAC TTC GAT GTC	F1	
43	GGA	GAC A	AA GGG TA		G GTT ACT AC		C CC		AC TGG TAC TTC GAT GTC	D8,9	
48	GGA	GAC AG	G A		TA GTA GCT AC		C		C TGG TAC TTC GAT GTC	F1	
V _H 12-J _H 4											
Productive											
16	GGA	G	TC A		AT GAT GGT TAC		AAT GT		C TAT GCT ATG GAC TAC	D9	VNDGYNVYAMDY
18	GGA	G	TC		GAT GGT TAC T		C		C TAT GCT ATG GAC TAC	D9	VDGYSYAMDY
26	GGA	GAC AGA	TC		G TTT TCC TC				C TAT GCT ATG GAC TAC	?	DRSFSSYAMDY
37	GGA	GAC			TC				C TAT GCT ATG GAC TAC	?	DSYAMDY
40	GGA	GAC AGA	TC		G GAT GGT TAC T		TC	T	AT TAC TAT GCT ATG GAC TAC	D9	DRSDGYFFYYAMDY
Non-productive											
1	GGA	G			TT ATT ACT ACG GTA GTA GC				TAC TAT GCT ATG GAC TAC	F1	
2	GGA				AC				ACT ATG GAC TAC	?	
3	GGA	G	G		G GTA G		G		GCT ATG GAC TAC	F1	
5	GGA	GAC AGA	T		AG AAT AGA CAG CTG GCG G				TG GAC TAC	?	
9	GGA	GAC AGA			TAG TA		G		GCT ATG GAC TAC	DX	
10	GGA	GAC AGA	TC	G AGG	GAT TAC		ACC	AT TAC TAT GCT ATG GAC TAC	D2		
8	GGA	GAC	C		TT TAT TAC TRAC GGTT AGT AG		G C	C TAT GCT ATG GAC TAC	F1		
11	GGA	GAC AGA	TC	C	TAT TAC TAC GGT AG		C CG	TAC TAT GCT ATG GAC TAC	F1		
13	GGA	GAC	GG		G ATG GTT ACT AC		G GG	AC TAT GCT ATG GAC TAC	D9		
17	GGA	GAC	TCT C		TCT ACT ATG ATT ACG ACG		GGG CTT GGG G	TAT GCT ATG GAC TAC	D2		
22	GGA	GAC AG			G GTT ACT		CCT ATG	CT ATG GAC TAC	D9		
24	GGA	GAC AGA	TC	T C	CT ATG ATT ACG AC		G GG	T AT TAC TAT GCT ATG GAC TAC	D2		
31	GGA	GAC A	CC CCC		ATA GTA ACT AC		T CT	T AT TAC TAT GCT ATG GAC TAC	DX		
36	GGA	GAC			CGC GGA AGG G			C TAT GCT ATG GAC TAC	?		
38	GGA	GAC AG	C TCA		GGC TAC AG			GCT ATG GAC TAC	F2		
39	GGA	GAC AGA	AA		A TAG TAA CT		T TAC CT	T TAC TAT GCT ATG GAC TAC	DX		
45	GGA	GAC AGA	T		AG TAA C			TAC TAT GCT ATG GAC TAC	DX		
47	GGA	GAC AGA	ATC CCA		TTT ATT ACT ACG GTA GTA GC		C CT	T AT TAC TAT GCT ATG GAC TAC	F1		
48	GGA	G			CC CCG T			T ATG GAC TAC	?		
49	GGA	GAC AGA	GGA		TAT GAT TAC GAC		GGG TAT TAG G	AT GCT ATG GAC TAC	D2		
51	GGA	GAC AGA	TC		G ATT ACG		TTC CTA TT	CT ATG GAC TAC	D2		
52	GGA	G	TC TTC CTC CC		T CTA TGA TGG TTA CT		C CC	C TAT GCT ATG GAC TAC	D9		

Fig. 1. CDR3 nucleotide sequences of V_H12-J_H1 and V_H12-J_H4 rearrangements in BM of 2^ab mice. Sequences were generated using pooled BM genomic DNA from three mice. The nucleotides are assigned to V, D and J genes, or to N and P nucleotide additions (Feeny, 1990; Roth *et al.*, 1992) based on the germline sequence of V_H12 (Clarke and McCray, 1993), J_H (Sakano *et al.*, 1980) and D genes (Kurosawa *et al.*, 1981, Kurosawa and Tonegawa, 1982; Lawlaer *et al.*, 1987; Alessandrini and Desiderio, 1991; Gu *et al.*, 1991b; Chang *et al.*, 1992; Feeny and Riblet, 1993). D gene use is indicated as follows: D1-11 or X, DSP2 genes; F1 or 2, DFL16 genes; Q, DQ52. For those D sequences which can come from multiple D genes, all possible D gene names were given and separated by commas. For D-D fusions, the D genes involved are separated by a slash. Unassigned D genes are indicated by question marks. Letters in lower case indicate mismatches to the relevant gene sequences and may represent PCR amplification errors. The last 31 nucleotides of V_H12 in the J_H4 clone 39 have been deleted.

The selective loss of non-10/G4, but not 10/G4, P rearrangements predicts an enrichment of 10/G4 sequences among P rearrangements. This is exactly what was observed (Figure 3). Among P rearrangements in pre-B cells, 19% (6/32) have a 10/G4 CDR3. There may also be an enrichment of rearrangements with a CDR3 of 10 amino acids without a Gly in the fourth position, as they account for 22% (7/32) of P rearrangements.

V_H12 rearrangements are diverse in the absence of selection

To exclude the possibility that the absence of most V_H12 P rearrangements and the enrichment of 10/G4

P rearrangements from pre-B cells is due to a biased rearrangement mechanism unique to V_H12, we examined V_H12-J_H1 rearrangements in BM of μ MT mice. μ MT mice cannot generate pre-BII or B cells because the membrane exon of the C μ gene is disrupted by homologous recombination (Kitamura *et al.*, 1991). Therefore, the V_HCDR3 repertoire in μ MT mice cannot be affected by μ chain-based selection. As shown in Figure 4, 34% (28/54) of rearrangements are in-frame, as expected from a random rearrangement process. Thus, the low frequency of P rearrangements among pre-BII cells in normal mice is not due to a biased rearrangement mechanism. Among the 22 P sequences in μ MT mice, two have a CDR3 of

Clone	V _H	P _V	N	P _D	D	P _D	N	P _J	J _H ¹	D	gene	a. a. seq.
Genomic DNA of sorted B cells												
Productive												
B9	GGA	GAC	AGA	GGG	AAA TAT G	GG	TAA CGA C	G	G G	AC TGG TAC TTC GAT GTC	D3,4,6	DRGKYLRRDWFVDV
B24	GGA	GAC		TC		A	ACT GG		T	TAC TGG TAC TTC GAT GTC	Q	DSTGYWYFDV
B16	GGA	G		CC	GCA	AAC	TGG		CCT	TGG TAC TTC GAT GTC	Q	AANPWYFDV
B18	GGA	GAC	AGA	AGG	GTT ACG	TAT	TAC TAC GGT AGT AGC TAC	GT	A CCT	TAC TGG TAC TTC GAT GTC	F1	DRRVTYGGSSYPYWFVDV
B42	GGA	GAC	AG	G	AG	C	GGT			TAC TGG TAC TTC GAT GTC	F1	DRSGWYFDV
B11	GGA	GA				T	TAC TAC GG			C TAC TGG TAC TTC GAT GTC	F1,2	DYGYWYFDV
B19	GGA	GAC	AGA			GGG	GGT			TAC TGG TAC TTC GAT GTC	?	DRGGYWYFDV
Non-productive												
B34	GGA	GAC		CC		C	TAC TAT GAT TAC GA			TAC TGG TAC TTC GAT GTC	D2	
B47	GGA	GAC	AGA			TCT	ACT ATG ATT ACG		TC	AC TGG TAC TTC GAT GTC	D2	
B2	GGA	GAC	AG	G	AAG	GGT	AAC T		T	TGG TAC TTC GAT GTC	D1,5,7,8	
B14	GGA	GAC	AGA	CCT	TT	T	ATG GTA ACT AC	G	CGG G	C TGG TAC TTC GAT GTC	D1,5,7,8	
B41	GGA	GAC	AGA			ACT	ATG GTA AC		C CC	TGG TAC TTC GAT GTC	D1,5,7,8	
B26	GGA	GAC	AGA			TAT	Ggc TAC GAC		AGG A	TAC TTC GAT GTC	D2,3,4,6	
B4	GGA	GAC	AGA	TC	C	ACT	ATA GTA AC		G TAG	GG TAC TTC GAT GTC	DX	
B29	GGA	GAC	A	T	CC	T	CTA CTA TAG TAA CTA C	GT	AGG G	TGG TAC TTC GAT GTC	DX	
B15	GGA	GAC	AG	G	G	AC	TGG GA		T TAC GC	AC TGG TAC TTC GAT GTC	Q	
B27	GGA	GAC	AGA	T	AA	G	TGG GAC	G	GG G	TGG TAC TTC GAT GTC	Q	
B35	GGA	GAC	AGA			AT	ACT ACG GTA GTA GCT		GCA GTA CC	TC GAT GTC	F1	
B36	GGA	GAC		C		TA	TIA CTA CGG TAG TAG		TCC T	C TAC TGG TAC TTC GAT GTC	F1	
B39	GGA	GAC		CCC		TTT	ATT ACT ACG GTA GTA GC		C	GG TAC TTC GAT GTC	F1	
B44	GGA	GAC				TAC	GGT AGT AGC TAC		CCC	C TAC TGG TAC TTC GAT GTC	F1	
B13	GGA	GAC	AGA	T	AG	AGG GG	A TTA CTA CGG TAG T			TAC TTC GAT GTC	F1	
B25	GG			G	CGA GGG	TTA	TIA CTA CGG TAG TAG CTA	GAG		TGG TAC TTC GAT GTC	F1	
B46	GG			G	GTG ATG G	TT	ACT ACG GTA	CC		TC GAT GTC	F1	
Genomic DNA of sorted pre-B cells												
Productive												
A47	GG			G	G	AT	AGT AAC TAC	G		G C TAC TGG TAC TTC GAT GTC	DX	DSNYGYWYFDV
A6	GGA	GA		A	AT	C	TGG G		GT	TAC TGG TAC TTC GAT GTC	Q	QINGWYFDV
A41	GGA	GAC	AG	G	GGG	TAC	TAC GGT AGT AGC		TTT	TAC TGG TAC TTC GAT GTC	F1	DRGYGSSPYWYFDV
B2	GGA	G		TC	CG	C	TAC GGT AGT AG		T TTC C	AC TGG TAC TTC GAT GTC	F1	VRYGSSFWYFDV
A48	GGA	GA		T	CT	T	ACG G		AC GTA G	GG TAC TTC GAT GTC	F1,2	DLTDVGYFDV
A37	GGA	GAC	AGA	AA		C	GGG			TAC TGG TAC TTC GAT GTC	Dst4	DRNGWYFDV
Non-productive												
A45	GGA	GAC		CA		T	ACT ATG ATT A		AG GG	AC TGG TAC TTC GAT GTC	D2	
B1	GGA	GAC	AGA			TAT	GAT TA		A C	C TGG TAC TTC GAT GTC	D2	
A13	GGA	GAC	AGA	T	AG	CC	C TAT GAT TAC G			AC TGG TAC TTC GAT GTC	D2	
A5	GGA	Gat	AG			G	TTT ACT AC		T TCC C	TAC TGG TAC TTC GAT GTC	D9	
A8	GGA	GAC	AGA			TGA	TGG TTA CTA C		A	G TAC TTC GAT GTC	D9	
A15	GGA	GAC		CT		T	GAT GGT TAC TAC		C	C TGG TAC TTC GAT GTC	D9	
A32	GGA	GAC	AGA	TC		G	ATG GT		C C	C TAC TGG TAC TTC GAT GTC	D9	
A21	GGA	GA		G		ACT	ATG ATT AGG			GG TAC TTC GAT GTC	D9	
A43	GGA	GA		A		CTA	TGA T		CT TAC CCC C	AC TGG TAC TTC GAT GTC	D9	
A40	GGA	GAC	AGA		GGG	TAT	AGT AAC		CTC	AC TGG TAC TTC GAT GTC	DX	
B11	GGA	GAC	AGA	T	AA	G	AGT AAC TAC	G	CC CGT A	AC TTC GAT GTC	DX	
B43	GGA	GAC	AGA	T	AG	G	AG TAA C	G	A GG	GG TAC TTC GAT GTC	DX	
A16	GGA	GAC	AaA	TC	T	G	TA TGG TAA			C TAC TGG TAC TTC GAT GTC	D1,5,7,8	
A7	GGA	GAC	AGA	T	TT	TGA	TAA CTA		GA	GG TAC TTC GAT GTC	D1,5,7,8	
A35	GGA	GAC	A		AG	AA	T ACG			AC TGG TAC TTC GAT GTC	D2-4,6,10	
A12	GGA	GAC	A			TA	ACT GGG AC	G	GAG G	TGG TAC TTC GAT GTC	Q	
B14	GGA	GAC	AGA	TC		C	TGG GAC	GT	T CC	GG TAC TTC GAT GTC	Q	
A52	GGA	GAC	A		AG	A	CT AA		G	C TGG TAC TTC GAT GTC	Q	
A4	GGA	GAC	A		CCC	ATT	ACT ACG GTA GTA GCT		T	TGG TAC TTC GAT GTC	F1	
A14	GGA	G		GC	CCT	TTA	CTA CGG TAG TAG		T	TGG TAC TTC GAT GTC	F1	
A17	GGA	GAC	AGA	T	GA	AGG	CTA CGG TAG TAG		TCCC	C TAC TGG TAC TTC GAT GTC	F1	
A26	GGA	GAC	AGA	T	TT	GAG	ACT ACG GTA GTA GCT AC	G	TA C	C TGG TAC TTC GAT GTC	F1	
A27	GGA	GAC		C		AT	TAC TAC GGT AGT AGC TAC		CC	TAC TTC GAT GTC	F1	
A38	GGA	GAC	A		CA	GGG G	CT ACG GTA G			GG TAC TTC GAT GTC	F1	
A42	GGA	GAC	AGA	TC	C	CCC	TCC			TAC TGG TAC TTC GAT GTC	F1	
A53	GGA	GAC	AGA		AAG		CTA CGG TAG TAG C		CC ACC AA	AC TGG TAC TTC GAT GTC	F1	
A57	GGA	GAC		CCC	TCG	GTC	TTA TTA CTA CGG TAG T		GG TGA	AC TTC GAT GTC	F1	
A59	GGA	GAC	AGA	TC	A	CGG	TAG TAG C		CT C	AC TGG TAC TTC GAT GTC	F1	
B7	GGA	GAC	AG	C	G	TT	ACT ACG GTA GTA GCT AC		T GA	GG TAC TTC GAT GTC	F1	
B9	GGA			GTC	G	T	TAC TAC GGT AGT A		CC T	C TAC TGG TAC TTC GAT GTC	F1	
333	GGA	GAC				TTA	TIA C		GT CTC	C TGG TAC TTC GAT GTC	F1	
49	GGA	GAC	AG	C	GAT	TGG	GTT ACT ACG GTA G		G	TAC TGG TAC TTC GAT GTC	Q/F1	
38	GGA	GAC				T	TIA TTA CTA CGG TAG TAG GGG A		AC TT	TAC TGG TAC TTC GAT GTC	F1/Q	
11	GGA	GAC	AGA		GGG	C	GGG CTA C	G	G CCC CTC	C TAC TGG TAC TTC GAT GTC	Dst4	
144	GGA	GAC	A			TA	CGG C		CC CTC	C TAC TGG TAC TTC GAT GTC	F2	
119	GGA	GAC	A			AG	AAG ACT T			TGG TAC TTC GAT GTC	?	
119	GGA	GAC	AGA	TC		C	CCT C			G TAC TTC GAT GTC	?	
13	GGA	GAC	A		A	G	GAC		TCG	AC TGG TAC TTC GAT GTC	Q	
cDNA of sorted pre-B cells												
2	GGA	GAC	AGA		G	AT	GAT TAC G		TC C	AC TTC GAT GTC	D2	DRDDYVHFDV
42	GGA	GAC	AGA			GAT	T		GT	TAC TGG TAC TTC GAT GTC	D2	DRDCYWFVDV
35	GGA	GAC	AGA			TAT	GAT GGT TAC T		CT	TAC TGG TAC TTC GAT GTC	D9	DRYDGYSSWYFDV
43	GGA	GAC	AGA		ACC	CCC	GG			C TAC TGG TAC TTC GAT GTC	D9	DRTPGSGWYFDV
66	GGA	GA		T		TCT	ATG ATG GTT ACT AC	G	GCC C	AT GTC	D9	DSMMVTIAHV
33	GGA			TG		T	GGT AAC			TAC gGG TAC TTC GAT GTC	D1,5,7,8	CGNYGYFDV
B49	GGA	G		TC	CC	G	TTA CGA C		CC GA	G TAC TTC GAT GTC	D3,4,6	VPFRPEYFDV
B60	GGA	GAC	AG		G	A	AT GGT			TAC TGG TAC TTC GAT GTC	D1,3-9	DRNGWYFDV
B5	GGA	GAC	AGA		AG		G GTT ACG TAT TAC TAC GGT AGT AGC TAC	GT	A CCT	TAC TGG TAC TTC GAT GTC	D3,4/F1	DRRVTYGGSSYPYWFVDV
B20	GGA	G		CC	CT		C TAT GT A			AC TGG TAC TTC GAT GTC	DX	ALYSNNWYFDV
B23	GGA	G		TC		G	CC TAC T		TT	AG C TAC TGG TAC TTC GAT GTC	D6-8,11,x	VAYFSWYFDV
B4	GGA	G		TC	CT		T AAC TGG G			AC TGG TAC TTC GAT GTC	Q	VLNWDWYFDV
B26	GGA	G		TC	CC	A	G CT AAC CTG GG		G GGG GGG	TAC TGG TAC TTC GAT GTC	Q	VPANLGGGYWYFDV
B73	GGA	GAC	AG		C		CT GG		T	TAC TGG TAC TTC GAT GTC	Q	DSLGWYFDV
B1	GGA	G		TC	ACG	GG	T TAC TAC GGT AGT AGC TAC		CCT	TAC TGG TAC TTC GAT GTC	F1	VTJYGGSSYWFVDV
B7	GGA	G		GG	GGC	CT	C TAC GGT AGT			TGG TAC TTC GAT GTC	F1	GGLYGSPWYFDV
B8	GGA	GAC		CCC	A		AC TAC GGT A			AC TGG TAC TTC GAT GTC	F1	DPNYGNWYFDV
B9	GGA	GAC				TAT	TAC TAC GGT AGT AGC			TAC TGG TAC TTC GAT GTC	F1	DPYYGSSYWFVDV
B11	GGA	GA				T	TAC TAC GGT A		AG G	GG TAC TTC GAT GTC	F1	DYGGKGYFDV
B30	GGA	G		TCG		T	TIA CTA CG		T G	G C TAC TGG TAC TTC GAT GTC	F1	VSLRGWYFDV
B34	GGA	G		TC		TAC	TAC GGT AGT AGC			TAC gGG TAC TTC GAT GTC	F1	VYGGSSGYFDV
B46	GG			T		T	T TAT TAC TAC GGT AGT AGC		C	GG TAC TTC GAT GTC	F1	IYYGSSRYFDV
B3	GGA	GAC	AGA		C		AC GGT AGT AGC			TAC TGG TAC TTC GAT GTC	F1	DRHGSSWYFDV
B50	GGA	G		TC	CG		C TAC GGT AGT AG		T TTC C	AC TGG TAC TTC GAT GTC	F1	VRYGSSFWYFDV
B12	GGA	GAC	AG			G	AGG GGT			GAC TGG TAC TTC GAT GTC	?	DRRGDWYFDV
B25						CGT				TAC TTC GAT GTC	?	YFDV

Fig. 2. CDR3 nucleotide sequences of V_H12-J_H1 rearrangements in sorted BM B and pre-B cells of 2^H4^b mice. Both genomic and cDNA were amplified for rearrangements in sorted pre-B cells, while rearrangements in sorted B cells were amplified in genomic DNA only. The origin of sequences for mouse A or B are indicated. The last nine nucleotides in clone B25 from pre-B cDNA are deleted. For other details, see legend to Figure 1.

CDR3 amino acid sequences of P rearrangements in pre-B cells of normal mice

Clone	Sequences	Length
B25	YFDV	4
B33	CGNYGYFDV	9
A37*	DRNGYMYFDV	10
A6*	QIWGYMYFDV	10
B11	DYYGKYFDV	10
B12	DRRGDMYFDV	10
B60	DRNGYMYFDV	10
B73	DSLGYMYFDV	10
B2	DRDDYVHFV	10
B4	VLNMDMYFDV	10
B20	ALYSNMYFDV	10
B42	DRDCYMYFDV	10
B49	VPFRPEYFDV	10
B66	DSMMVTAAHV	10
A48*	DLTDVGYFDV	10
B8	DPNYGNMYFDV	11
B23	VAYFSYMYFDV	11
A47*	DSNYGYMYFDV	11
B7	GGLYGSPLYFDV	12
B30	VSLLRGYMYFDV	12
B34	VYYGSSYGYFDV	12
B46	IYYGSSRYFDV	12
B3	DRHGSSYMYFDV	12
B2*	VRYGSSPHMYFDV	13
B43	DRTPGDGYMYFDV	13
B50	VRYGSSPHMYFDV	13
B35	DRYDGYMYFDV	13
B9	DPYYGSSYMYFDV	14
B26	VPANLGGYMYFDV	14
B1	VTGYGSSYMYFDV	15
A41*	DRGYGSSYMYFDV	15
B5	DRRVYYGSSYVPYMYFDV	20

CDR3 amino acid sequences of P rearrangements in μ MT BM

66	DSLLGLDV	8
37	DRGYMYFDV	9
10	DRQYNMYFDV	10
71	QYRYKTYFDV	10
31	DYDGYDMYFDV	11
20	QNLVLKGYFDV	11
90	DRSTIYMYFDV	11
78	DRNYGYMYFDV	11
9	DRLTGYMYFDV	11
42	DYGSYGYMYFDV	12
B2	DSMVTTPMYFDV	12
B20	DRMGLRPYMYFDV	13
113	DRGTMVQYMYFDV	13
110	DKGYPYGNMYFDV	14
41	DKRYDGSDMYFDV	14
84	DRLGYNGYMYFDV	14
93	DRSDRYGPHMYFDV	14
111	DGTYYGSSYMYFDV	14
B25	DGGYYGDDHYMYFDV	15
14	DRGYGSSYDHYMYFDV	15
95	DRFITVTIYMYFDV	16
39	QDYGSSPSTMVYMYFDV	18

Fig. 3. Comparison of V_H12 – J_H1 CDR3 sequences in BM pre-B cells of normal mice and BM of μ MT mice. Sequences are listed according to CDR3 length. CDR3s of 10 amino acids are boxed and the 10/G4 CDR3s are underlined. All sequences are taken from Figures 2 and 4. For pre-B cells of normal mice, sequences are combined from both cDNA and genomic DNA (indicated by asterisks).

10 amino acids, and none has a 10/G4 CDR3 (Figure 3), indicating that the occurrence of a 10/G4 P rearrangement is rare. This is different from the frequency of 10/G4 among pre-BII cells of normal mice ($P = 0.035$ by χ^2). Although the frequency of P rearrangements that have a CDR3 of 10 amino acids but do not have a Gly in the fourth position is lower in μ MT (2/22 or 9%) than in pre-BII in normal mice (7/32 or 22%), the difference has not reached statistical significance ($P = 0.15$ by χ^2). Thus, neither the absence of most V_H12 P rearrangements, nor enrichment of 10/G4 P rearrangements among pre-BII cells in normal mice, is the result of a biased rearrangement mechanism.

Both 10/G4 and non-10/G4 μ chains can associate with SL and be expressed on the pre-B cell surface

As μ chains must associate with SL and be expressed on the surface for normal development of pre-B cells, we determined whether V_H CDR3 affects association and expression of V_H12 μ chains. For this purpose, constructs encoding three non-10/G4 V_H12 μ chains were generated.

One construct, 8/G0, has a CDR3 sequence based on the DSP2.2 gene sequence but was not observed among P rearrangements. The CDR3s of the other two constructs, 12/G3.7 and 14/G7, have sequences from DFL16.1 and DSP2.9 D genes, respectively, and were observed among P rearrangements in the BM of μ MT mice. These constructs, and the original 10/G4 construct (Arnold *et al.*, 1994), were transfected into a μ chain loss variant of pre-B cell line, Bine 4.8. Cell lysates of G418-resistant transfectants were screened by an anti- μ enzyme-linked immunosorbent assay (ELISA) and positive transfectants with similar levels of μ expression were metabolically labeled with [35 S]Met/Cys and immunoprecipitated with anti- μ . Two proteins with apparent molecular weights of 21 and 15 kDa were associated with all four μ chains (Figure 5) and were identical in size to $\lambda 5$ and VpreB from a Bine 4.8 cell line transfected with a V_HJ558 gene (data not shown). These μ -positive transfectants were also stained with anti- μ and analyzed by FACS. All express μ chains on the cell surface (Figure 6). Therefore, the absence of non-10/G4 P rearrangements among pre-BII cells cannot be ascribed to an inability to form a μ /SL complex.

Discussion

To determine when non-10/G4 B cells are lost from the repertoire, we analyzed V_H12 rearrangements in BM. Surprisingly, we find that the loss of V_H12 -expressing cells is evident among pre-B cells, and that the mechanism responsible is V_H CDR3 sequence dependent. Thus, while ~95% of pre-BII cells with non-10/G4 P rearrangements are absent, we estimate, from the enrichment of 10/G4 P rearrangements among pre-B cells, that there is little or no loss of 10/G4 V_H12 -expressing pre-B cells. A rearrangement mechanism responsible for the biased repertoire is ruled out since, in μ MT mice, where μ chain-based selection cannot occur, there is no enrichment of 10/G4 P rearrangements and the frequency of P and NP rearrangement is as expected for a random rearrangement process. We have also verified that non-10/G4 μ chains can associate with SL and be expressed on the pre-B cell surface. Thus, we conclude that selection of V_H12 B lineage cells is based on the V_H CDR3 sequence and occurs during the transition from the pre-BI to pre-BII cell stage. This dependence on V_H CDR3 sequence for cell survival argues that production and cell surface expression of a μ /SL receptor by itself does not guarantee inclusion in the pre-BII cell compartment.

We have suggested previously that the restricted CDR3 repertoire of mature V_H12 B cells in adult and neonatal mice is due to positive selection (Clarke and McCray, 1993; Haughton *et al.*, 1993; Ye *et al.*, 1995); B lineage cells must bind ligand to complete differentiation and, in the absence of the ligand binding and subsequent signal transduction, they undergo PCD. The present study indicates that such positive selection would operate at the pre-BII cell stage when the μ chain is expressed on the cell surface with the SL chain. Consistent with this idea, a requirement for signal transduction through the μ /SL receptor is indicated by the arrest of B cell development at the transition between the pre-BI and pre-BII cell stages in μ MT, $\lambda 5T$ (Kitamura *et al.*, 1991, 1992) and RAG

Clone	V _H	P _V	N	P _D	D	P _D	N	P _J	J _{H1}	D gene	CDR3 a.a.
In-frame=28											
B20	GGA GAC AGA		A TG G		GA TTA CGA C		CT		TAC TGG TAC TTC GAT GTC	D2	DRMGLRPLYWYFDV
5*	GGA GAC AGA	T	AG GG		C TAT GAT TAC GA		G TT		C TAC TGG TAC TTC GAT GTC	D2	
A2*	GGA GAC		TCC		CTA CTA TGA TTA CG		G		TAC TGG TAC TTC GAT GTC	D2	
10	GGA GAC AGA		G		AG TA		C A		AC TGG TAC TTC GAT GTC	DX	DRQYNWYFDV
20	GGA GA		A AAT T		TA GT		C CTA AAG G		GG TAC TTC GAT GTC	DX	QNLVLKGYFDV
110	GGA GAC A		AG GGC C		CC TAC TAT GGT AAC		A		AC TGG TAC TTC GAT GTC	D7	DKGPYYGNWYFDV
96*	GGA GA		G CC		C CTA CTA TGG TAA C		T		C TAC TGG TAC TTC GAT GTC	D7	
31	GGA GAC				TAT GAT GGT TAC G				AC TGG TAC TTC GAT GTC	D9	DYDGYDWYFDV
41	GGA GAC A		AG AGG G		TC TAT GAT GGT T		CC G		AC TGG TAC TTC GAT GTC	D9	DKRVYDGSWYFDV
84	GGA GAC AGA		C		TC TAT GGT TAC GAC	G		G	C TAC TGG TAC TTC GAT GTC	D3,4,6	DRLYGYNGWYFDV
90	GGA GAC AGA	TC	G		ACT AT		T		TAC TGG TAC TTC GAT GTC	D1-8,10,11	DRSTLYWYFDV
71	GGA GA		A		TAT AGG TAC		AAG AC		G TAC TTC GAT GTC	D10,11	QYRYKTYFDV
93	GGA GAC AGA	TC	G G		AT AGG TAC G		GT CCT C		AC TGG TAC TTC GAT GTC	D10,11	DRSDRYGPHWYFDV
B2	GGA GAC		T		C T ATG GTA ACT AC		C CC		C TGG TAC TTC GAT GTC	D1,5,7	DSMVTTPWYFDV
78	GGA GAC AGA				AAC TAC	G		G	C TAC TGG TAC TTC GAT GTC	D1,5,7,8	DRNYYGYWYFDV
113	GGA GAC AGA		GG		T ACT ATG GTA ACT		CAA		TGG TAC TTC GAT GTC	D1,5,7	DRGTMTWQWYFDV
66	GGA GAC AG		C		CTG GGN C				TC GAT GTC	Q	DSLLGLDV
9	GGA GAC AG			G	CTA ACT GGG				TAC TGG TAC TTC GAT GTC	Q	DRLTGYWYFDV
B25	GGA GAC		GGA GGG		TAT TAC TAC GGT AGT AGC		C		AC TGG TAC TTC GAT GTC	F1	DGGYYGSDHWYFDV
14	GGA GAC AGA		GGG		TAC TAC GGT AGT AGC TAC	G			AC TGG TAC TTC GAT GTC	F1	DRGYGSSVWYFDV
42	GGA GAC				TAC GGT AGT AGC TAC		GG		TGG TAC TTC GAT GTC	F1	DYSSSYGWYFDV
95	GGA GAC AGA				TTT ATT ACT ACG GTA GTA GCT A		T		C TAC TGG TAC TTC GAT GTC	F1	DRFITTVTVIYWYFDV
111	GGA GAC		GGG AC		T TAC TAC GGT AGT AGC				TAC TGG TAC TTC GAT GTC	F1	DGTYGSSVWYFDV
117*	GGA GAC		CG		T TAT TAC TAC GGT AGT AGC T		GA GGC CT		C TAC TGG TAC TTC GAT GTC	F1	
39	GGA GAC		C CAG ACA GAG G		AC TAC GGT AGT AGC CCC TCT				C TAC TGG TAC TTC GAT GTC	F1/D1,5	QDYGSSPSTMTWYWYFDV
					ACT ATG GTA AC						
37	GGA GAC AGA				GGG				TAC TGG TAC TTC GAT GTC	?	DRGYWYFDV
2*	GGA GAC A		AG		CTA CTA TGG TAA CAA CGG G		TG		TAC YGG TAC TTC GAT GTC	D1,5,7/Dst4	
107*	GGA GAC AGA	T	AG G		TA CTA CGG C		TC G		AC TGG TAC TTC GAT GTC	F2	
Out of-frame=54											
23	GGA G		GC GGC AA		G ATT AC				GG TAC TTC GAT GTC	D2	
49	GG				TCT ACT ATG GTT ACG AC		A GGG AAC			D2	
51	GGA GAC AGA	TC	G GG		A CTA TGA TTA CGA C	GT	CCT TCC CC		GG TAC TTC GAT GTC	D2	
6	GGA G		TC CTT AT		C CTA CTA TGG TAA		GGG GG		GG TAC TTC GAT GTC	D7	
3	GGA GAC		T		TA TGA TGG TTA				C TAC TGG TAC TTC GAT GTC	D9	
17	GGA GA				T ATG ATG GTT AC				TAC TGG TAC TTC GAT GTC	D9	
89	GGA G		GA CAG GG		G ATG GTT AC		C T		C TGG TAC TTC GAT GTC	D9	
119	GGA GAC A		C		G ATG GTT ACT A		A		GG TAC TTC GAT GTC	D9	
101	GGA GAC A				TG ATG GTT ACT AC	G			AC TGG TAC TTC GAT GTC	D9	
108	GGA GAC AGA	TC	G GGG G		TA CTA			G	C TAC TGG TAC TTC GAT GTC	D9	
116	GGA GA		T		CCT ACT ATA GGT		C		C TAC TGG TAC TTC GAT GTC	D11	
60	GGA GAC		C		CC TAC TAT AGG TAC		CT		TAC TGG TAC TTC GAT GTC	D11	
106	G				TA CGA CGT GGG				C TAC TGG TAC TTC GAT GTC	D10,11/Q	
122	GGA GAC AGA	T			TG GGG GTA GTA GC		A AG		TGG TAC TTC GAT GTC	Q/F1	
59	GGA GAC				GTA C		CC T		C TAC TGG TAC TTC GAT GTC	D10,11	
105	GGA GAC AGA		CC		T AGG				GG TAC TTC GAT GTC	D10,11	
73	GGA G		CT GG		C TAT AGG TAC		TT		TAC TGG TAC TTC GAT GTC	D10,11	
29	GGA GAC AGA	TC	T CC		A CTA TAG G		GG		AC TGG TAC TTC GAT GTC	D10,11	
44	GGA GAC AG		G		ACT ATA GGT ACG AC	G	G		C TGG TAC TTC GAT GTC	D10,11	
B26	GGA GAC A		AT AA		C TAC TAT AGG TAC GAC				C TGG TAC TTC GAT GTC	D10,11	
B41	GGA GAC AGA	TC	C		TAT AGG TAC GAC	GT	G GGG AAC T		AC TGG TAC TTC GAT GTC	D10,11	
69	GGA GAC AGA				CTA CTA TGG TAA				C TAC TGG TAC TTC GAT GTC	D1,5,7	
100	GGA GA		G T		TA TGG TAA C				TAC TGG TAC TTC GAT GTC	D1,5,7,8	
7	GGA GAC AGA	TC	C CCC CCC CCT AG		G GTA ACT AC		C C		C TGG TAC TTC GAT GTC	D1,5,7,8	
1	GGA GAC		T		TA TGG TAA C		C		AC TGG TAC TTC GAT GTC	D1,5,7,8	
56	GGA GAC AGA		G		AC TAT		CT		TAC TGG TAC TTC GAT GTC	D1-8,10,11	
11	GGA GAC AGA		GTC TT		T ATG GTT ACG		GA T		C TAC TGG TAC TTC GAT GTC	D3,4	
12	GGA GAC AG		T AT		T ATG G		CC CT		TGG TAC TTC GAT GTC	D1,3-8	
68	GGA GAC A				TG GTT				C TAC TGG TAC TTC GAT GTC	D1,3,4,6-9	
72	GGA GAC AGA				GGG TTA CGA C		C		C TGG TAC TTC GAT GTC	D3,4,6	
75	GGA GAC AGA	TC			A TGG TTT TAC TAC GGT AGT AGC TAC	GT	C TTC		AC TGG TAC TTC GAT GTC	D3,4,6,9/F1	
77	GGA GAC AG		G CCC GA		A TTA CTA CGG TAG TAG CTA CTG GTA		G		TAC TGG TAC TTC GAT GTC	F1/D1,5,7,8	
4	GG		C CGG GGA A		GG GTA C		TC C		C TGG TAC TTC GAT GTC	Dst4	
13	GGA GAC AGA	TC	G CG		T ATT ACT ACG GTA GTA GCT A		GG TC		C TAC TGG TAC TTC GAT GTC	F1	
16	GGA GgC AG		G CCC C		TAT TAC TAC GGT AGT AGC		A		TAC TGG TAC TTC GAT GTC	F1	
A1	GGA GAC AGA	TC	C T	A	T TTA TTA CTA CGG TAG TAA				C GAT GTC	F1	
B30	GGA GAC AG		T C		TT ATT ACT ACG GTA GTA GCT AC		C		GG TAC TTC GAT GTC	F1	
112	GGA GAC AG		G GG		T ACT ACG GTA GT		G AA		TAC TGG TAC TTC GAT GTC	F1	
103	GGA GAC AGA		AA		A TAC TAC GGT AGT AG				TAC TGG TAC TTC GAT GTC	F1	
92	GGA GAC AGA	TC	C TG		A TTA CTA CGG TAG TAG CTA C		G	G	C TAC TGG TAC TTC GAT GTC	F1	
57	GGA G		TC CTC TC		A TTA CTA CGG TAG TAG		A		TGG TAC TTC GAT GTC	F1	
85	GGA GAC AGA	T	GT GG		T ACT ACG GTA GTA GC		C TC		AC TGG TAC TTC GAT GTC	F1	
50	GGA GAC A		CC GG		A CGG TAG TAG		GGG		AC TGG TAC TTC GAT GTC	F1	
67	GGA GAC AG		C GCC GA		A TTA CTA CGG TAG TAG C		CC CTC		AC TGG TAC TTC GAT GTC	F1	
18	GGA GAC AGA	TC	C CAG ATC GG		T ATT ACT ACG GTA GTA GCT AC		C TTC		AC TGG TAC TTC GAT GTC	F1	
22	GGA GAC		TCC		TAT TAC TAC GGT AGT AGC		CC		C TAC TGG TAC TTC GAT GTC	F1	
30	GGA GA		C CCT T	A	T TTA TTA CTA CGG TAG TA		C C	G	C TAC TGG TAC TTC GAT GTC	F1	
34	GGA		CCC T		TT ACT ACG GTA GTA G		TC		AC TGG TAC TTC GAT GTC	F1	
65	GGA GAC AG		T C	A	T TTA TTA CTA CGG TAG TAG CT		C		C TAC TGG TAC TTC GAT GTC	F1	
45	GGA GAC AGA	T	G		A TTA CTA CGG TAG TAG CTA C		CC CCA CCT		GAT GTC	F1	
					TAT TTT A		CC				
74	GGA GAC		C		TA TTA CTA CGG T				C TAC TGG TAC TTC GAT GTC	F1	
27	GG		C C		TA CTA CGG				C TAC TGG TAC TTC GAT GTC	F2	
94	GGA GAC		CCC CAA TTC		ATT ACT ACG GC		C T		C TGG TAC TTC GAT GTC	F2	
53	GGA GAC AGA	TC			T CST GAC CGG				GG TAC TTC GAT GTC	?	

Fig. 4. CDR3 nucleotide sequences of V_H12-J_H1 rearrangements in BM of μ MT mice. Sequences of A and B series, respectively, were generated from unsorted BM cells of mouse A and B. The rest of the sequences were generated from sorted B220⁺ cells in pooled BM from 4 μ MT mice. In-frame rearrangements with an asterisk include a stop codon in the D region and are therefore NP rearrangements. The last 10 nucleotides of V_H12 and the first 23 nucleotides of J_H1 are deleted in clone 39 and in clone 49, respectively. Because of their length, the D sequence and N sequence for clone 39 and clone 45, respectively, are given on two lines. For other details, see legend to Figure 1.

knock-out mice (Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992). Furthermore, a H chain Tg generating a μ /SL receptor can restore normal development of pre-BII cells in RAG gene knock-out mice (Spanopolou *et al.*, 1994; Young *et al.*, 1994). The μ /SL receptor presumably can also mediate proliferative expansion of pre-BII cells (Decker *et al.*, 1991b; Osmond, 1991; Kitamura *et al.*,

1992). For V_H12-expressing pre-BII cells, we suggest that only those with certain CDR3 sequences bind ligand and continue differentiation, and that most fail to bind ligand and die by PCD. Positively selected V_H12 pre-B cells would clearly include those with a 10/G4 V_HCDR3 sequence, since they are enriched in the pre-B population. Although not yet statistically significant, positively

Fig. 5. Association of V_H12 μ chains with $\lambda 5$ and $V_{pre}B$. Pre-B cell line Bine 4.8 transfected with V_H12 μ chain constructs were labeled with [^{35}S]Met/Cys and precipitated with either rabbit anti-mouse μ or normal rabbit γ -globulin (NR γ G). Precipitates were run on a 12% SDS-acrylamide gel. The CDR3 amino acid sequences are DYDGYWYFDV (10/G4), DYYDYFDY (8/G0), DYGSYGWYFDV (12/G3,7) and DKRVYDGSWYFDV (14/G7).

selected cells may also include those with V_H CDR3 sequences of 10 amino acids without the Gly in the fourth position, as they constitute 22% of the pre-B cell P repertoire and are highly represented in the neonatal liver (Clarke and McCray, 1993). Thus, there may be a hierarchy of CDR3 sequences with 10/G4 superior to all others.

Approximately 75% of cytoplasmic μ^+ pre-B cells are lost in the adult BM, suggesting that cell death at this transition stage is common (Osmond, 1991). This model also offers a simple explanation for the elimination of pre-B cells expressing a $D\mu$ /SL receptor (Gu *et al.*, 1991b). Such cells would not be able to bind ligand due to the absence of a V_H domain and, thus, could not be rescued from PCD. The inability of cells that have rearranged both *Igh* alleles non-productively to make the transition from pre-BI to pre-BII would be due to the same lack of a rescue signal.

The alternative explanation for the selective loss of V_H12 -expressing pre-BII cells that we cannot exclude is negative selection, as is the case with self-reactive T and B cells. However, this would probably require multiple selecting ligands and is a more complex model, since there appears to be no single selective structural basis for the elimination of these cells that would not also eliminate those with the 10/G4.

Although non-10/G4 μ chains are incapable of facilitating B cell development, they do, however, appear to mediate allelic exclusion. This is borne out by the extremely low non-10/G4 P/NP observed in both BM pre-B and B cell populations. Had non-10/G4 P V_H12 rearrangements, like NP V_H12 rearrangements, not excluded further V_H -D- J_H rearrangement, then non-10/G4 P rearrangements could be rescued by subsequent P rearrangements on the other allele. In this case, non-10/G4 P rearrangements would not be selectively eliminated

Fig. 6. Expression of V_H12 μ chains on the surface of pre-B cells. Transfectants representing μ chains with different CDR3s were stained with FITC-labeled goat anti-mouse μ (thin line) or no antibody (thick line) and analyzed by FACSscan.

from the repertoire, and the non-10/G4 P/NP would be that generated by random rearrangement (0.37) as seen in μ MT mice (Figure 4). This is not what we observed. The combined data from all BM populations give a non-10/G4 P/NP of 0.15 (11/73) (Table I, J_H1 rearrangements), significantly lower than that observed in μ MT mice ($P = 0.025$ by χ^2). Thus, non-10/G4 μ chains must mediate allelic exclusion. As the membrane form of the μ chain is required for both allelic exclusion (Kitamura and Rajewsky, 1992) and pre-BII cell survival (Kitamura *et al.*, 1991), this suggests that two distinct signals are delivered by the μ chain, one to mediate allelic exclusion and the other to rescue pre-B cells from PCD. In this regard, it is interesting to note that while $D\mu$ does not support pre-B cell development, it appears to mediate allelic exclusion (Corcos *et al.*, 1991; Ehlich *et al.*, 1994). Thus, the V_H region appears to be necessary for survival of pre-BII cells, but not allelic exclusion.

V_H -dependent selection of pre-B cells is not likely to be unique to those expressing V_H12 . The P/NP for individual V_H genes varies over a wide range. For example, the V_H81X gene exhibits a P/NP among BM pre-B cells of 0.33–0.42 (Decker *et al.*, 1991a; Huetz *et al.*, 1993), similar to that of V_H12 -expressing pre-B cells, while the P/NP among BM pre-B cells is ~ 6 for the M315 V_H gene of the V_H36 –60 family (Decker *et al.*, 1991b). The wide range could be due to differing selective pressures on each V_H region. These differences could relate ultimately to how each V_H region interacts with ligand and the availability of ligand. Some V_H regions, like the M315 V_H region, may interact with ligand primarily through CDRs 1 and 2 and have little contact through CDR3. In this case, M315 V_H -expressing pre-B cells, regardless of the V_H CDR3 sequence, could bind ligand and be positively selected. 81X V_H regions may have significant contact through CDR3, resulting in selection of only a subset of pre-B cells expressing this gene, as we have proposed for V_H12 . Although no V_H CDR3 restriction is apparent among V_H81X -expressing pre-B cells, there are few P rearrangements available for comparison. The detection of CDR3

restriction among V_H12-expressing B cells is facilitated by strong subsequent clonal expansion in the periphery (Pennel *et al.*, 1989; Arnold *et al.*, 1994; Ye *et al.*, 1995). For B cells expressing other V_H genes, CDR3 restriction may be difficult to discern, if there is no subsequent clonal expansion or comparison with an unselected population (Decker *et al.*, 1991a,b; Huetz *et al.*, 1993).

The function of V_H-dependent selection at the pre-BII stage is not clear, although it could ensure a functional repertoire of V_H regions. It is interesting to note that the 10/G4 heavy chains are used primarily in PtC-specific B cells, which exist in various species including humans (Alving, 1984; Kawaguchi, 1991), and V_H12 and V_H11, both of which contribute to the anti-PtC specificity (Hardy *et al.*, 1989; Pennel *et al.*, 1989), are much more conserved than other V_H genes over evolutionary time (Booker and Haughton, 1994). Therefore, V_H-dependent selection at the pre-BII cell stage may serve to ensure production of not only B cells with a functional H chain, but also B cells with a physiologically useful H chain.

Materials and methods

Mice

B10.H-2^H-4^bp/Wts (2^a4^b) mice were maintained in our mouse colony at the University of North Carolina at Chapel Hill. μ MT mice (Kitamura *et al.*, 1991) were kindly provided by Dr Derry Roopenian at the Jackson Laboratory (Bar Harbor, ME).

Cell sorting

BM single cell suspensions were prepared in Hank's balanced salt solution containing 0.1% sodium azide and 1% fetal calf serum (FCS). Cells were stained with biotinylated anti-B220 (RA3-6B2) and FITC-labeled anti-IgM^b (AF6-78) (Pharmingen, San Diego, CA). Streptavidin-PE (Jackson Immuno-Research Laboratories, West Grove, PA) was used to reveal biotinylated antibody. Cell sorting was done using an EPICS 751 flow cytometer (Coulter Electronics, Hinleah, FL). Pre-B cells and B cells were sorted as B220⁺/surface IgM⁻ and B220⁺/surface IgM⁺, respectively. Re-analysis was made using a FACScan (Becton-Dickinson, Mountain View, CA) equipped with acquisition computer and software from Cytomation (Fort Collins, CO). Sorted pre-B cells contain <1% IgM⁺ cells and sorted B cells contain <3% pre-B cells. To estimate the contamination by plasma cells, 1 × 10⁴ sorted pre-B cells were transferred onto slides, fixed in solution containing 0.02% Na₂HPO₄, 0.1% KH₂PO₄, 10% formaldehyde and 45% acetone, stained with FITC-labeled anti- κ (Pharmingen, San Diego, CA) and examined under a fluorescence microscope. No plasma cells were observed after scanning of at least 500 cells.

PCR, cloning of V_H12 rearrangements and sequencing

DNA extraction, PCRs, cloning of PCR products and sequencing were described previously (Clarke and McCray, 1993). Due to scarcity of B lineage cells and therefore templates of V-D-J rearrangements in the BM from μ MT mice, DNA extracted from B220⁺-enriched cells was used for PCR. For this purpose, BM cells from μ MT mice are stained with

biotinylated anti-B220 antibody, followed by addition of streptavidin-coupled Dynabeads M-280 magnetic beads (DynaL, Lake Success, NY). B220⁺ B cells were then collected using a magnetic tube holder.

For cDNA synthesis, the Micro-FastTrack mRNA Isolation kit (Invitrogen, CA) was used to extract poly(A)⁺ RNA. cDNA synthesis was initiated with a primer. C μ (5'-GCAGGAGACGAGGGGGA-3'), complementary to the coding region of the C μ 1 gene, using a cDNA Cycle kit (Invitrogen, CA). J_H1E (5'-TGAGGAGACGGTGACCGTGGTC-3') primer and V_H12 FWR1 primer (Clarke and McCray, 1993) were used for amplification of V_H12-J_H1 rearrangements from cDNA.

Construction of the μ chain expression vectors

The 10/G4 expression vector (CDR3 amino acid sequence: DYDGYWYFDV) was described previously as V_H12.CH27-C μ (Arnold *et al.*, 1994). Other expression vectors with different CDR3s were generated by modifying the 10/G4 vector by PCR using a set of overlapping primers (Table II). The CDR3 nucleotide sequence for 8/G0, GACTACTATGATTCTTCGATGTC, is based on the DSP2.2 gene but has not been observed among P rearrangements. The CDR3s of the other two constructs, 12/G3.7 and 14/G7, were taken from the CDR3 sequences of μ MT clones 42 and clone 41, respectively (Figure 4).

For construction of the 8/G0 expression vector, three sequential PCRs were performed using the 10/G4 construct as a template to generate a fragment that includes the V exon with a new CDR3 sequence and the 5' non-coding DNA containing the promoter region of the original clone. In each PCR, the same 5' primer (V_H12-4) that anneals to DNA 308 bp 5' of the V_H12 leader exon and includes a *Kpn*I restriction site (Table II) was used along with three different 3' primers to create a new CDR3. The 10/G4 vector was used as the template in the first PCR with the V_H12-4 primer and a 3' primer (V_H12-1), complementary to the 3' end of V_H12 and carrying the sequence for a new CDR3 including the 5' end of the J_H1 gene. In the second PCR, the purified product of the first PCR was used as the template. The 3' primer (V_H12-2) was complementary to the 3' end of V_H12-1 and contained additional DNA of the J_H1 gene.

Fig. 7. V_H12 μ chain constructs. (A) 8/G0, (B) 12/G3.7 and 14/G7. Restriction sites are as follows: S, *Sal*I; N, *Not*I; B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; Sf, *Sfi*I.

Table II. Nested primers used for the construction of μ chain expression vectors

Primers	Sequence (5' to 3')
V _H 12-1	CCACAGGTGCAAGTAATCATAGTAGTCTCCTGCACAGTAATACATGGC
V _H 12-2	AACGGTGACCGTGGTCCCTGCGCCCCAGACATCGAGGTAATCATAGTAGT
V11-3	TGAAGTCTAGACTTACCTGAGGAAACGGTGACCGTGGTCCCTGCGCC
V _H 12-4	CCGAGCTCGAATTCACCTAGAAATTGAA
V _H 12-5N	GAATTCACCTAGCGGCGCAAAATTGAAGGTAGGATTAAACCAAGC
V _H 12-3N	ATTTTGAAAATGCGGCGCTAAATACATTTTGAAGTCGATAAACT
D12-3	GTACCACCCGTAGCTACTACCGTAGTCTCCTGCACAGTAATACATGGC
D12-5	TACGGTAGTAGCTACGGGTGGTACTTCGATGCTCGGGGCACAGG
D14-3	GTCGGAACCATCATAGACCCCTTGTCTCCTGCACAGTAATACATGGC
D14-5	AAGAGGGTCTATGATGGTCCGACTGGTACTTCGATGCTCGGGGCACAGG

Complete J_H1 sequences were added by amplifying purified product of the second PCR using a 3' primer (V11-3). This final 3' primer contained an *Xba*I restriction site. The final PCR product was cloned into TA cloning vector (Invitrogen, San Diego, CA), released by *Kpn*I-*Xba*I restriction and subcloned into the *Kpn*I-*Xba*I site of a PBS vector containing the C_μ enhancer. The 1.5 kb *Eco*RI fragment containing the V-D-J coding region including its 5' sequence and C_μ enhancer was then subcloned into the *Eco*RI site of pSV2-Neo-MPL vector (Arnold *et al.*, 1994). An *Eco*RI C_μ fragment of 12.8 kb was isolated from the 10/G4 vector (Arnold *et al.*, 1994) and cloned into the *Eco*RI site of pSV2-Neo-MPL containing the V-D-J insert.

12/G3,7 and 14/G7 vectors were generated using a 'splicing by overlap extension' strategy (Clackson *et al.*, 1991). In the first PCR, two fragments with 24 bp overlapping nucleotides in the CDR3 region were generated. One fragment was generated using the 10/G4 vector as template with a 5' primer (V_H12-5N), complementary to DNA 307 bp 5' of V_H12 leader exon and a 3' primer (D12-3 for 12/G3,7 and D14-3 for 14/G7), complementary to a sequence to the 3' end of the V_H12 coding region and carrying the new CDR3 sequence (Table II). The other fragment was generated using 8/G0 as template with a 5' primer (D12-5 for 12/G3,7 and D14-5 for 14/G7), complementary to the new CDR3 and the 5' end of the J_H1 coding region sequence, and a 3' primer (V_H12-3N), spanning the *Eco*RI site 3' of the C_μ enhancer. These two overlapping fragments were purified and joined together in the second PCR using the V_H12-5N and V_H12-3N oligonucleotides. The 1.5 kb joined fragments were cloned into the TA cloning vector. As both the V_H12-5N and V_H12-3N primers have *Not*I sites, the 1.5 kb insert was released conveniently with *Not*I digestion and subcloned to the *Not*I site of the pSV2-Neo-MPL-C_μ vector. Clones with inserts in the correct orientation were identified with restriction digest analysis. The structures of the μ chain expression vectors are shown in Figure 7.

Transfection

Approximately 1×10⁷ Bine 4.8 cells (a kind gift from Dr H. Martin-Jack, Loyola University of Chicago, Maywood, IL), a μ chain loss variant of the pre-B cell line 18-81, were washed twice with phosphate-buffered saline (PBS) and resuspended in a Gene Pulser cuvette (0.4 cm electrode) in 0.45 ml of PBS with 10 μg of plasmid constructs linearized with *Sfi*I. Electroporation was done using a Bio-Rad Gene Pulser apparatus (Hercules, CA) at 500 μF, 0.25 kV. Cells were then transferred to 24-well plates (Costar, Cambridge, MA) at ~4×10⁵/well and selected in DMEM-H medium supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1× non-essential amino acids, 100 U/ml penicillin and streptomycin, and 0.6 mg/ml G418 (Gibco, Grand Island, NY). G418-resistant colonies were screened by anti-μ ELISA of cell lysates. None of the μ⁺ transfectants expresses κ or λ conventional light chains as determined by ELISA. For detection of surface μ chain, Bine 4.8 transfectants were stained with FITC-labeled goat anti-mouse μ antibody (Fisher Scientific, Pittsburgh, PA) in a volume of 30 μl. Stained cells were analyzed using a FACScan (Becton-Dickinson, Mountain View, CA) equipped with Cytomation software (Fort Collins, CO).

ELISA

Goat anti-mouse μ, κ or λ antibodies (Southern Biotechnology Associates, Birmingham, AL) were diluted in carbonate buffer (pH 9.6) and coated on ELISA plates (Costar, Cambridge, MA) at 100 μl/well by incubating overnight at 4°C. Excess antibody was washed away with PBS containing 0.2% Tween. One hundred microlitres of lysing buffer [20 mM NaCl, 6 mM MgCl₂, 20 mM Tris pH 7.4, 0.02% phenylmethylsulfonyl fluoride (PMSF) and 1% NP-40] were added to each well, followed by addition of 3–5×10⁴ cells in a volume of 100 μl. Cells were mixed well and incubated for 4 h at room temperature. Plates were then washed with PBS containing 0.2% Tween, and alkaline phosphatase-conjugated goat anti-mouse μ, κ or λ (Southern Biotechnology Associates, Birmingham, AL) were added and incubated for 4 h. Paranitrophenyl phosphate (Sigma, St. Louis, MO) was added to each well after the final wash and read at 405/600 nm dual wave length.

Immunoprecipitation

The cells (5×10⁶) were washed twice in PBS, resuspended in 1 ml of Cys/Met-free RPMI (Gibco, Grand Island, NY) supplemented with 10% dialyzed FCS, and cultured at 37°C for 1 h. Cells were labeled by adding 200 μCi of [³⁵S]Met/Cys (Amersham, Arlington Heights, IL) and culturing for an additional 2 h. Labeled cells were lysed for 30 min in 0.5% NP-40 lysing buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 5 mM iodoacetamide; 2 mM PMSF). Cell lysates were pre-cleared with

protein A-Sepharose beads (Pharmacia, Uppsala, Sweden). Pre-cleared cell lysates were precipitated at 4°C for 1 h with addition of affinity-purified rabbit anti-mouse μ antibody or normal rabbit γ-globulin (Jackson Immuno-Research Laboratories, West Grove, PA) as a negative control, and collected on protein A beads. Immunoprecipitates were washed, resuspended in 60 μl of Laemmli buffer, boiled for 5 min, and run on 12% SDS-acrylamide gels. Molecular standards were pre-stained SDS-PAGE standards (Bio-Rad Laboratories, Hercules, CA) and Rainbow molecular weight markers (Amersham, Arlington Heights, IL). The gels were dried, exposed to a PhosphorImager screen, and analyzed using a PhosphorImager (Molecular Dynamics).

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