Immunity, Vol. 4, 133–144, February, 1996, Copyright ©1996 by Cell Press

Surrogate Light Chain Expression Is Required to Establish Immunoglobulin Heavy Chain Allelic Exclusion during Early B Cell Development

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Summary

Allelic exclusion at the IgH locus was examined in B lineage cells of wild-type mice and mice unable to express the surrogate light chain molecule λ5 using a single-cell PCR approach. By analyzing B precursor cells containing two $V_HD_HJ_H$ rearrangements, we found that in wild-type animals, cells are allelically excluded as soon as μ chains are expressed. Furthermore, we provide evidence that in cells expressing Dμ proteins $V_H \rightarrow D_HJ_H$ rearrangement is inhibited. In contrast, in the absence of λ5 protein, B precursor cells were allelically "included," indicating that allelic exclusion at the IgH locus requires expression of the pre-B cell receptor either containing a μ chain or a Dμ chain. However, although μ chain double-producing B precursor cells are generated in λ5-deficient mice, such cells were not detected among surface immunoglobulin positive B cells.

Introduction

B cell antigen receptor genes are assembled by a program of somatic gene rearrangements from variable region genes (V_H), diversity genes (D_H), and joining elements (J_H) at the immunoglobulin heavy chain (IgH) loci and from V_L and J_L genes at the immunoglobulin light chain (IgL) loci, respectively (reviewed by Tonegawa, 1983). At the IgH locus, first D_HJ_H rearrangements are assembled on both chromosomes, and this is followed by $V_H \rightarrow D_HJ_H$ joining (Alt et al., 1984). If the $V_H \rightarrow D_HJ_H$ rearrangement creates an open reading frame, i.e., is productive, a heavy chain of class μ is expressed. Although B lineage cells can undergo $V_HD_HJ_H$ rearrangements on both IgH loci, only one of both IgH alleles is expressed by a B cell, i.e., B cells are allelically excluded.

Several models have been proposed to account for allelic exclusion. The stochastic model suggests that allelic exclusion of IgH loci is due to a low probability to yield two productive $V_HD_HJ_H$ rearrangements in a cell within a given time period (Coleclough et al., 1981; Walfield et al., 1981; Langman and Cohn, 1987; Cohn and Langman, 1990). In a second model, Wabl and Steinberg (1982) proposed that expression of both IgH chain loci is toxic for the respective cell and leads to the death of μ chain double producers. While these models assume that $V_H \rightarrow D_HJ_H$ joining proceeds independently at both alleles, Alt et al. (1981, 1984) postulated that μ chain expression from a productive $V_HD_HJ_H$ rearrangement prevents further IgH gene recombination (Figure 1). This ordered model of rearrangement, initially based on work using transformed B cell precursors, is supported by studies with μ chain transgenic and gene knockout mice. First, μ chain transgenic mice show greatly decreased levels of $V_HD_HJ_H$ rearrangements at the endogenous IgH loci, suggesting an inhibitory effect of the transgene-encoded μ chain on endogenous $V_H \rightarrow D_HJ_H$ joining (Rusconi and Köhler, 1985; Weaver et al., 1985; Manz et al., 1988; Nussenzweig et al., 1988). Second, B cells of mice carrying a disrupted membrane exon of the μ chain on one IgH allele (μMT mice, Kitamura et al., 1991) show allelic inclusion of the targeted IgH chain locus, suggesting that expression of a membrane-bound μ chain generates a signal leading to inhibition of $V_H \rightarrow D_HJ_H$ recombination at the IgH locus (Kitamura and Rajewsky, 1992).

With respect to the B cell precursor population, the ordered model would predict that cells are allelically excluded already at very early developmental stages; namely, as soon as they express μ chains. Our previous analysis (Ehlich et al., 1994) showed that $CD43^-$ precursors are indeed allelically excluded; however, that study did not address this question for cells at the preceding $CD43^+$ stage.

By studying transformed pre-B cell lines, it was discovered that the μ chain associates with a surrogate L chain encoded by the two genes *VpreB* and *λ5* (Pillai and Baltimore, 1987; Karasuyama et al., 1990; Takemori et al., 1990; Nishimoto et al., 1991; Tsubata and Reth, 1990). This so-called pre-B cell receptor can be expressed on the cell surface (Tsubata and Reth, 1990; Lassoued et al., 1993; Karasuyama et al., 1993; Winkler et al., 1995) and is active in signal transduction (Tsubata et al., 1992). This has led to the hypothesis that μ chain–mediated allelic exclusion requires the assembly of the μ chain and the surrogate L chain into a pre-B cell receptor complex (Figure 1; Rolink and Melchers, 1991; Rajewsky, 1992; Bauer and Scheuermann, 1993).

Early pre-B cells can also express a second form of a pre-B cell receptor that contains a truncated μ chain lacking the V_H domain (Reth and Alt, 1984). This so-called Dμ protein is expressed from D_HJ_H rearrangements in one of the three possible D_H reading frames (reading frame [RF] 2 in the nomenclature of Ichihara et al., 1989) and associates with λ5 and VpreB as conventional μ chains do (Tsubata et al., 1991). Interestingly, D_H elements joined in the Dμ protein encoding RF are rarely found in D_HJ_H and $V_HD_HJ_H$ complexes of surface immunoglobulin $(sIg)^-$ pre-B cells and peripheral B cells (Meek, 1990; Gu et al., 1991a; Kaartinen and Mäkelä, 1985). Whereas most productive $V_HD_HJ_H$ joints of peripheral B cells contain D_H genes joined to J_H segments in RF1, RF1 and RF3 are equally utilized in nonproductive $V_HD_HJ_H$ joints. This underrepresentation of RF3 in productive joints is due to stop codons present in most D_H elements if read in that frame (Kaartinen and Mäkelä, 1985; Gu et al., 1991a). B cells of heterozygous μMT mice (Kitamura et al., 1991) show no counterselection against RF2 in D_HJ_H joints on the targeted H chain allele (Gu et al.,

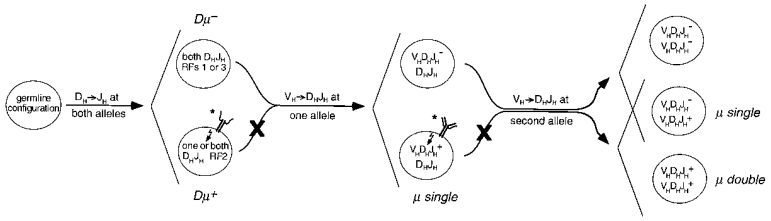

Figure 1. Patterns of IgH Gene Rearrangements during B Cell Development

According to the ordered model, expression of a Dμ or conventional μ chain inhibits $V_H \rightarrow D_H J_H$ joining, leading to the suppression of pathways marked by a cross. Asterisk, pre-B cell receptor, containing a Dμ (left) or μ (right) chain. IgL chain gene rearrangement is not considered in this figure.

1991a). From this result, Gu and coworkers (1991a) hypothesized that cells expressing membrane-bound Dμ proteins are impeded in further development because the Dμ protein, similar to a conventional μ chain, prevents $V_H \rightarrow D_H J_H$ recombination (Figure 1), as had been earlier suggested in a more general way by Reth et al. (1985) and Lennon and Perry (1985). Thus, Dμ protein expressing B cell progenitors would provide a model system to study allelic exclusion at the IgH locus.

Mutant mice lacking either a (membrane-bound) μ chain or the λ5 component of the pre-B cell receptor show a severe block in early B cell development, indicating that this receptor complex is essential in guiding early differentiation steps in B cell development (reviewed by Löffert et al., 1994). If exclusion of the second IgH allele also is achieved through pre-B cell receptor signaling, one would predict that allelic exclusion is not established in precursor B cells lacking pre-B cell receptor expression. Surprisingly, however, Kitamura et al. (1992) found that peripheral B cells of λ5-deficient mice are allelically excluded. Thus, either the hypothesis of pre-B cell receptor–mediated allelic exclusion is incorrect, or, in contrast with the situation in wild-type mice, double-producing precursor cells are indeed generated in λ5-deficient mice but do not enter the pool of mature B cells.

We have addressed this issue of allelic exclusion in the B cell precursor compartment by analyzing single cells for their IgH gene rearrangements. Furthermore, to answer the question whether the second form of the pre-B cell receptor (containing a Dμ protein) is indeed capable of signaling allelic exclusion, we followed the usage of the Dμ protein encoding RF at various stages of B cell development by the analysis of $D_H J_H$ and $V_H D_H J_H$ rearrangements isolated by polymerase chain reaction (PCR) from B lineage cell populations. By using wild-type and λ5-deficient mice in these analyses, we were able to distinguish between the role of the μ chain (or Dμ protein) and its respective pre-B cell receptor for allelic exclusion at the IgH locus.

Results

Early B Progenitor Cells Are Allelically Excluded Already at the CD43$^+$ Stage

The analysis of IgH gene rearrangements at the level of individual cells (Ehlich et al., 1994; Ehlich and Küppers, 1995) allows us to address directly the question whether allelic exclusion at the IgH locus is established by μ chain expression. Using multicolor flow cytometry, single cells were isolated from the precursor compartment in which IgH gene rearrangements become first detectable. In the flow cytometric system developed by Hardy et al. (1991), sIg$^-$ B cell precursors in mouse bone marrow are resolved into CD43 expressing B progenitor cells and CD43$^-$ pre-B cells. CD43$^+$ B cell progenitors can be further dissected into fractions A, B, C, and C′ according to the expression of cell surface markers CD45R/B220, CD43/S7, HSA, and BP-1 (Hardy et al., 1991). Whereas at the CD43$^-$ pre-B cell stage all cells already contain productive $V_H D_H J_H$ joints, the CD43$^+$ compartment comprises cells undergoing $D_H \rightarrow J_H$ and $V_H \rightarrow D_H J_H$ rearrangements (Ehlich et al., 1994). It has been suggested that CD43$^-$ (or CD43^{lo}) cells undergo rapid proliferation before they differentiate into small resting pre-B cells (Karasuyama et al., 1993). Therefore, to exclude the possibility of counterselection of μ chain double producers during such a proliferative phase, we isolated single progenitor cells that are still at the CD43$^+$ stage (cells of fraction C [B220$^+$CD43$^+$HSA$^+$BP-1$^+$] where cells have been detected that contain $V_H D_H J_H$ rearrangements at both chromosomes; Ehlich et al., 1994). Cells carrying productive $V_H D_H J_H$ rearrangements were enriched by isolating single cytoplasmic μ$^+$ cells. From these cells, $V_H D_H J_H$ and $D_H J_H$ joints were amplified by PCR and sequenced. The sequences representing rearrangements of those cells carrying two $V_H D_H J_H$ joints are shown in Table 1.

Assuming that the distribution of productive and nonproductive $V_H D_H J_H$ joints is random in B cell progenitors, 14% of the μ$^+$ cells containing two $V_H D_H J_H$ rearrangements would be expected to express μ chains from both IgH loci (taking into account that one-third of the joints carry the V_H gene sequence in frame with the J_H element, and that approximately 80% of the D_H genes encode stop codons when joined to a J_H element in RF3 [Gu et al., 1991a]; note that the predicted number of double producers might vary between 11%–14% depending on whether RF2 counterselection is taken into consideration). In contrast with this expectation, all 21 B progenitor cells that contained two $V_H D_H J_H$ rearrangements carried only one productive $V_H D_H J_H$ joint (Table 2).

It thus appears that allelic exclusion at the IgH locus is established early in B cell development, as soon as μ chains are expressed.

Allelic Inclusion at the IgH Locus in Early B Progenitor Cells of Mice Unable to Express λ5 Protein

To investigate whether allelic exclusion at the IgH locus can be established by μ chains alone or whether this process requires also the presence of the surrogate L chain, we examined $V_H D_H J_H$ rearrangements in B cell progenitors of λ5-deficient mice isolated from the same cellular fraction that was analyzed in wild-type mice (cytoplasmic μ$^+$ cells of fraction C).

Table 1. Sequences of $V_HD_HJ_H$ Junctional Regions in B Cell Precursors of λ5T Mice and C57BL/6 Mice as well as Splenic B Cells of λ5T Mice

3′V_H	P, N	D_H	P, N	J_H	V_H	D_H	D_H/J_H	±	RF
Bcf4									
1. TGT GCA AGA	ATC GG	C TAC GGT AGT AGC TAC	G	GG TTT GCT TAC TGG	VH1	DF116.1	J3	+	1
2. TGT GCA AGA	G ACG	ATT ACT ACG GTA GTA GCT AC	G TGG C	CT ATG GAC TAC TGG	VH12	DF116.1	J4	−	2
Bcf5									
1. TGT GCA AGA			TGG GGA GGG CTC CT	C TAT GCT ATG GAC TAC TGG	VH9	?	J4	+	?
2. TGT GC			TC GAA TAG CAA G	CC TGG TTT GCT TAC TGG	VH8	?	J3	−	?
Bcf30									
1. TGT GCA	G	AT TAC TAC GGT AGT AG	G	TTT GAC TAC TGG	VH1	DF116.1	J2	+	1
2. TGT GCA AGA	C GGG AGG GG	A CTA TGA TTA CG	T CCT G	CC TGG TTT GCT TAC TGG	VH12	DSP2.2	J3	−	3
Bcf65									
1. TGT NCA AGA		GGT AG	G GG	T TAC TAT GCT ATG GAC TAC TGG	VH1	DF116.1	J4	+	1
2. TGT GCA AGA	CC TCT TCT	CTG GGA C	GA GG	C TAC TGG	VH5	DQ52	J2	−	
Bcf70									
1. TGT GCA AGA	TGG	TGA TTA CGA C	GT CC	C TAC TGG	VH1	DSP2.2	J2	−	3
2. TGT GCA AGA	TTA GGA A	TC TAC TAT GAT TAC	CC	C TTT GAC TAC TGG	VH9	DSP2.2	J2	+	1
Bcf82									
1. TGT G	TG AGA ACG G	AC TAC GGT AG	C CC	G TTT GCT TAC TGG	VH6	DF116.1	J3	+	1
2. TGT GCA AG	GT	GAT TAC	TTC	TAC TAT GCT ATG GAC TAC TGG	VH1	DSP2.2	J4	−	1
Bcf85									
1. TGT GCA AGA			GGG GAC CTT TCG AAC CAG G	AC TTT GAC TAC TGG	VH1	?	J2	+	?
2. TGT GCA AGA	GA	TAT GGT TA	A C	AC TAC TGG	VH10	DSP2.3,4,6	J2	−	1
Bcf92									
1. TGT NCA AGA				TT GCT TAC TGG	VH1	?	J3	−	?
2. TGT GCA AGA		TAT GAT TAC G		AC TAT GCT ATG GAC TAC TGG	VH1	DSP2.2	J4	+	(1)
Bcf98									
1. TGT NC			CA AAA CNC TAC CCT AAG G	AC TAT GCT ATG GAC TAC TGG	VH2	?	J4	−	?
2. TGT ACA AGA			CCC	TAC TGG	VH5	?	J2	+	?
Bcf114									
1. TGT GCA	T	TA CGG TAG TAG CT	C C	TT GAC TAC TGG	VH1	DF116.1	J2	−	3
2. TGT GCA AGA			CAG A	AC TGG TAC TTC GAT GTC TGG	VH5	?	J1	+	?
Bcf129									
1. TGT GCA AG	G A	AT TAC TAC GGT AGT AGC TAC	TCT	TTT GCT TAC TGG	VH1	DF116.1	J3	+	1
2. TGT GCA AGA	C ATA AC	T CTA TGA TGG TT	G	GAC TGG	VH5	DSP2.9	J2	−	3
Bcf138									
1. TGT GCA AG			C G	AA GGG ACT	VH1	?	J3	+	?
2. TGT GCA AGA	T GGG	GGT AAC TAC	GA	T TAC TGG	VH3	DSP2.1,5,7,8	J3	−	1
Bcf2.6									
1. TGT GCA AGA	GAC CC	T TAT TAC TAC GGT AGT AGC TAC		TGG	VH1	DF116.1	J2	+	(1)
2. TGT GCA AGA			CA TGT GGG GTT TTA C	TT GAC TAC TGG	VH5	?	J2	−	?
Bcf2.15									
1. TGT GCC AGA			TCT GCT A	CC TGG TTT GCT TAC TGG	VH2	?	J3	+	?
2. TGT GCA AGA	A GAA GGG GTA A	TC TAC TAT		TTT GAC TAC TGG	VH4	DSP2.1–5,10	J2	−	1
Bcf2.18									
1. TGT GCA	GGA GAC AG	A TTA CTA CGG TAG TAG	AGG TTT C	AT GCT ATG GAC TAC TGG	VH12	DF116.1	J4	−	3
2. TGT GCA AGA	GAG G	TC TAC TAT G	CC C	GG TAC TTC GAT GTC TGG	VH5	DSP2.1–5	J1	+	1
Bcf2.26									
1. TGT GCA AGA		T TAC TNC GGT AGT AG	G T	AC TAC TTT GAC TAC TGG	VH1	DF116.1	J2	−	1
2. TGT ATG AGA		TAT GGT AGT AGC		TAC TGG TAC TTC GAT GTC TGG	VH11	DF116.1	J1	+	(1)
Bcf2.42									
1. TGT GCA AG	T	TGG GA	G GGG TGG TTA AAG GAG G	AT GCT ATG GAC TAC TGG	VH1	DQ52	J4	+	
2. TGT GCA AGA	C GGG	GAT GGT TAC TAC	G	AC TAT GCT ATG GAC TAC TGG	VH4	DSP2.9	J4	−	1

(*continued*)

Table 1. (*continued*)

3′V_H	P, N	D_H	P, N	J_H	V_H	D_H	D_H/J_H	±	RF
Bcf2.97									
1. TGT GCA AGA	TCC A	TC TAC TAT GAT TAC GAC	GGG A	TT GCT TAC TGG	VH1	DSP2.2	J3	+	1
2. TGT GCA AGA	T TT	T GGT AAC TAC	CCT C	CT ATG GAC TAC TGG	VH3	DSP2.1,5,7,8	J4	−	1
Bcf2.99									
1. TGT GCA AGA	TCC A	AC TAT GAT TAC GAC	GTG GGG	GCT ATG GAC TAC TGG	VH3	DSP2.2	J4	+	1
2. TGT GCA AGA	CC CC	T AGT AAC TAC	TCA GGG	GAC TAC TGG	VH5	DSP2.x	J2	−	1
λ5cf4.4									
1. NGN GCC AGA	AGG G	GA TGG TTA CTA C	GC CCC ACG	GCT ANG GAC TAC TGG	VH2	DSP2.9	J4	+	3
2. TGT GCA AGA	GG	T ACT ACG GTA GTA NC	C G	AC TGG TAC TTC GAT GTC TGG	VH9	DF116.1	J1	+	2
λ5cf4.10									
1. TGT	AGA G	CT ATG GTA ACT AC	C TTT	TAT GCT ATG GAC TAC TGG	VH6	DSP2.1,5,7	J4	+	2
2.	G GGG	GGT AGT AGC TAC	CAG A	AC TAC TGG	VH1	DF116.1	J2	−	1
λ5cf28									
1. TGT NCA AG	G G	GG TAA CNA C	GA	TTT GCT TAC TGG	VH1	DSP2.1,5,7,8	J3	−	3
2. TGT GCC AGA	GGG G	TA TTA CTA CG	C CT	C TAC TTT GAC TAC TGG	VH2	DF116.1	J2	+	3
λcf33									
1. TGT NCA AG	G AC	T ATT ACT ACG GTA GTA	TCT	GGC	VH10	DF116.1	J3	+	2
2. TGT GCA	TA A	CT ATG ATG GTT ACT AC	G CCT G	GG TTT GCT TAC TGG	VH1	DSP2.9	J3	−	2
λ5cf47									
1. TGT GCC A	AA CCC CG	C TGG GA	G G	AC TAT GCT ATG GAC TAC TGG	VH2	DQ52	J4	+	
2. TGT TCA ANA	CAG GGN	CTA TGG TAA CT	T TTT	TTT GAC TAC TGG	VH5	DSP2.1,5,7	J2	−	3
λ5cf52									
1. TGT GCA AGA	GGG GGG G	AC TAT GGT AAC TA	T T	AC TAC TTT GAC TAC TGG	VH5	DSP2.1,5,7	J2	+	1
2. TGT GCA AGA	TG GGG	GGT AGT ANC	CC	C TGG	VH1	DF116.1	J2	−	1
λ5cf62.1									
2. TGT GCC A	AA GGG A	TC TAC TAT GGT AAC TAC	G	AC TTT GAC TAC TGG	VH2	DSP2.1,5	J2	+	1
2. TG		ATG GTT ACT AC	G	TTT GAC TAC TGG	VH5	DSP2.9	J2	−	2
λ5cf78									
1. TGT GC	T CAA ATC NCC GAT CTG A	TC TAT GAT GGT	TTC TC	G TTT GCT TAC TGG	VH9	DSP2.9	J3	+	1
2. TGT GCA	C CTC	CTA TGA TTA C	TT TTT T	AT TAC TAT GCT ATG GAC TAC TGG	VH5	DSLP2.2	J4	−	3
λ5cf80									
1. TGT GCA AGA	CAT ATA TGT CCC C	CT ACT ATA GTA ACN AC	G TGG G	GG TTT GCT TAC TGG	VH5	DSP2.x	J3	+	2
2. TGT GCG AGA	T GGG G	TC NAC TAT GAT TA	A N	AC TTT GAC TAC TGG	VH9	DSP2.2	J2	−	1
λ5cf92									
1. TGT GCA A	CC ACA	TCT ACT ATG ATT ACG AC	A G	AC TGG TAC TTC GAT GTC TGG	VH1	DSP2.2	J1	+	2
2. TGT NCA AG	G GGG GGA G	CT GGG AC	G G	AT GCT ATG GAC TAC TGG	VH1	DQ52	J4	+	
λ5cf97									
1. TGT GC	C AAA AAC GG	C GGT AGT AGC	CCC T	AT TAC TAT GCT ATG GAC TAC TGG	VH2	DF116.1	J4	+	1
2. TGT GC	TC GAA	TAG CT	T AC	G TTT GCT TAC TGG	VH8	DF116.1	J3	−	3
λ5cf98									
1. TGT NCA AGA	TCC CA	T ATG ATG	ATA	TAC TTT GAC TAC TGG	VH1	DSP2.9	J2	+	2
2. TGT GCA AG	G CTA A	TT TAT TAC TAC GGT AGT AGC TAC	TTT T	CC TGG TTT GCT TAC TGG	VH5	DF116.1	J3	+	1
λ5cf127									
1. TGT	TCC AGN CCC AN	T TAC CAC GGT AGT AG	G AAC CNT	TTT NCT TAC TGG	VH5	DF116.1	J3	+	1
2. TGT GCG AGA	TG	G ATT ACG	GGA CGG C	TT GCT TAC TGG	VH9	DSP2.2	J3	+	2
λ5cf133									
1. TGT GCA AGA	AAG GGC CTT A	TT TAT TAC TAC GGT AGT AGC TAC	G	GG TAC TTC GAT GTC TGG	VH1	DF116.1	J1	+	1
2. TGT GCC AG			TT CCC TCT TCT CTT	TAC TGG	VH2	?	J2	−	?
λ5cf135									
1. TGT GC	TC GAA GGA AGA AGG T	TG ATG GTT ACT A	AA AGG	GCT TAC TGG	VH8	DSP2.9	J3	−	2
2. TGT GC	T CGA ATC CCA GGA CAG CTC AGG	CTA TG		C TTT GAC TAC TGG	VH8	DSP2.1–7	J2	+	3

(*continued*)

Table 1. (*continued*)

$3'V_H$	P, N	D_H	P, N	J_H	V_H	D_H	D_H/J_H	±	RF
λ5cf158									
1. TGT ACA AGA	AAG GGG GGG GAG TTT	TTA CTA	GAC GGC ACT G	GG TAC TTC GAT GTC TGG	VH1	DF116;DSP2.9	J1	+	3
2. TGT GCG AGA	TTG GG	T CTA CTA TGG TTA CG	G G	AC TGG TAC TTC GAT GTC TGG	VH9	DSP2.3,4	J1	+	3
λ5sp11									
1. TGT NCA AGA	GG	ACT ACG GTA GTA GC	A C	AC TAC TTT GAC TAC TGG	VH1	DF116.1	J2	−	2
2. TGT GCA AGA	CAT G	TG ATT ACG AC	G G	AT GCT ATG GAC TAC TGG	VH5	DSP2.2	J4	+	2
λ5sp12									
1. TGT GCA AGA		CTA CGG TAG TAG CTA C	TG T	AC TAC TTT GAC TAC TGG	VH1	DF116.1	J2	−	3
2. TGT	ACC ATC C	TC TAC TAT	AAC CG	T TAC TAT GCT ATG GAC TAC TGG	VH6	DSP2.1–5,10	J4	+	1
λ5sp3.1									
1. TGT GCA AGA	GGA	ATG ATT AC	C T	CT ATG GAC TAC TGG	VH1	DSP2.2	J4	+	2
2. TGT	AGT AGG AAG GA	A TAG TAA CTA C	TA A	AT TAC TAT GCT ATG GAC TAC TGG	VH13	DSP2.x	J4	−	3
λ5sp3.2									
1. TGT GCA AGA	GAG AGT CAT	TAT AGT A		AC TGG TAC TTC GAT GTC NGG	VH3	DSP2.x	J1	+	(1)
2. TGT GCA AGA	C	A TGA TGG TTA CT	C T	AC TAC TTT GAC TAC TGG	VH5	DSP2.9	J2	−	3
λ5sp4.2									
1. TGT GCA AGA	GG GGG AG	C TAC GGT AGT	T	CC TGG TTT GCT TAC TGG	VH1	DF116.1	J3	−	1
2. TGT NCA AGN	GGC CC	A CTA TGG		GCT ATG GAC TAC TGG	VH9	DSP2.1,3–7	J4	+	3
λ5sp1									
1. TGT NCA A			CT G	GG TTT GCT TAC TGG	VH1	?	J3	+	?
2. TGT		AC TAT AGT AAC		TAC TAT GCT ATG GAC TAC TGG	VH10	DSP2.x	J4	−	(1)
λ5sp5.11.4									
1. TGT GCA AGA	GA GGG G	GA TTA CGA C	AG GG	C TTT GAC TAC TGG	VH1	DSP2.2	J2	−	3
2. TGT GCC AGA	AGC CC	C TAC TAT AGT AAC		TAC TGG TAC TTC GAT GTC TGG	VH2	DSP2.x	J1	+	(1)
λ5spb.14									
1. TGT NC	C AAA	TAC TAC GGT AGT AG		C TAT GCT ATG GAC TAC TGG	VH2	DF116.1	J4	+	(1)
2. TGT GCA AGA			TGA	TTT GCT TAC TGG	VH5	?	J3	−	?
λ5spb1									
1. TGT GCA AGA	AAG GG	T ACT ATA GTA ACT	TT	C TAT GCT ATG GAC TAC TGG	VH1	DSP2.x	J4	+	2
2. TGT GCA AGA	T AGA TCT C	CT ACG GT	C GTT TCT TTT	TAT GCT ATG GAC TAC TGG	VH9	DF116.1	J4	−	2
λ5spb5									
1. TGT GCA AGA		TCT ACT ATG GTA A		CT ATG GAC TAC TGG	VH1	DSP2.11,5	J4	+	2
2. TGT GCA A				GCT TAC TGG	VH7	?	J3	−	?
λ5spN1									
1. TGT NCC A	CCA CC	T AGT AG		C TAT GCT ATG GAC TAC TGG	VH2	DF116.1	J4	−	(1)
2. TGT GCA AGA	AAA	ACT ATG GTA AC	C CCC GG	T GCT ATG GAC TAC TGN	VH9	DSP2.1,5,7	J4	+	2
λ5spb27									
1. TGT GC	G GGA	GAT GGT TAC TAC	GAG	GCT ATG GAC TAC TGG	VH1	DSP2.9	J4	+	1
2.	GG TGC TTG GAG AGA GCA A	AC TAT GGT AAC TAC	GGG	TAT GCT ATG GAC TAC TGG	VH8	DSP2.1,5,7	J4	−	1

Cells are designated by a letter denoting the cellular source and mouse strain (Bcf: fraction C, C57BL/6 mice; λ5cf: fraction C, λ5T mice; λ5sp: splenic B cells, λ5T mice) from which they were isolated, and a code number. The two $V_HD_HJ_H$ joints of a cell are arbitrarily numbered 1 and 2. V_H gene sequences were identified by comparison with V_H genes published by Kabat et al. (1991), D_H genes were assigned to published D_H segments, summarized by Chang et al. (1992), if there was homology of at least 5 nt. Nucleotides that can be either assigned to D_H genes or J_H segments are underlined. N and P nucleotides (Alt and Baltimore, 1982; Lafaille et al., 1989) are listed under P, N. A functional $V_HD_HJ_H$ joint is represented by a plus, and nonfunctional by a minus. RF indicates the D_H reading frame that was determined according to the nomenclature of Ichihara et al. (1989). IgH gene rearrangements that lack N nucleotides are predominantly joined to J_H in RF1 due to the presence of short regions of sequence homology at the border of the combining D_H and J_H elements (Gu et al., 1990; Feeney, 1990). Such D_HJ_H and $V_HD_HJ_H$ joints (numbers in parenthesis) were omitted from the RF calculations in the text.

Table 2. Numbers of B Lineage Cells Carrying Two $V_HD_HJ_H$ Rearrangements Isolated from λ5T Mice and C57BL/6 Wild-Type Mice

Cells	Mouse strain	VDJ^+/VDJ^-	VDJ^+/VDJ^+
$CD43^+$ $c\mu^+$ pro-B cells (fraction C)	C57BL/6	21*	0
	λ5T/λ5T	11	5
Splenic B cells	λ5T/λ5T	12	0

Data are summarized from Table 1. The absence of double producers from the $CD43^+$ B cell progenitor compartment of wild-type mice is statistically significant as is shown by a p value below 0.05 (Fischer's exact test). Asterisk, including two cells previously described by Ehlich et al. (1994) and V. Martin and K. R. (unpublished data), respectively.

If the surrogate L chain molecule λ5 is essential for μ chain–mediated allelic exclusion, in λ5-deficient mice about 14% of the μ^+ cells containing two $V_HD_HJ_H$ joints should be μ chain double producers (see above). Experimentally, we found 5 potential double producers among 16 progenitors that harbored two $V_HD_HJ_H$ joints (Table 2; Table 1, cells λ5cf4.4, λ5cf92, λ5cf98, λ5cf127, λ5cf158).

This finding indicates that allelic exclusion at the IgH locus requires coexpression of a μ chain and the surrogate L chain component λ5 in early B progenitor cells. We do not have a good explanation why we found, in the λ5-deficient mice, more cells bearing two in-frame $V_HD_HJ_H$ joints than theoretically expected (5 instead of 2). If it were due to statistical variation, this result would have a probability of around 5%.

sIg^+ B Cells of λ5-Deficient Mice Are Allelically Excluded

In contrast with the frequent observation of progenitor B cells in λ5-deficient mice containing productive $V_HD_HJ_H$ rearrangements at both IgH alleles, Kitamura et al. (1992) did not detect double-producing mature B cells by cytoplasmic staining of lipopolysaccharide-activated splenic B cells for the μ chain allotype in that mouse mutant. Since it cannot be excluded that this system selects against double producers, we directly analyzed ex vivo $V_HD_HJ_H$ rearrangements of single $B220^+sIgM^+$ splenic B cells of λ5-deficient mice by single-cell PCR (see Table 1).

Each of 12 splenic B cells containing two $V_HD_HJ_H$ rearrangements carried only one productive rearrangement, while the second $V_HD_HJ_H$ joint was nonproductive (Table 2). This result suggests that, in contrast with the situation in the progenitor compartment, splenic B cells of λ5-deficient mice are allelically excluded at the IgH locus.

To assess the question at which developmental stage double-producing B lineage cells are eliminated in the mutant animals, we examined newly generated B cells ($B220^+sIgM^+sIgD^-$) in the bone marrow of λ5-deficient and wild-type mice. Newly generated B cells are thought to be the direct descendants of $CD43^+$ progenitor cells in λ5-deficient mice, because this mouse mutant essentially lacks $CD43^-$ B cell precursors (Ehlich et al., 1993). The analysis was carried out by staining sIg on newly generated B cells in wild-type and λ5-deficient mice heterozygous for the μ chain allotypes *a* and *b*.

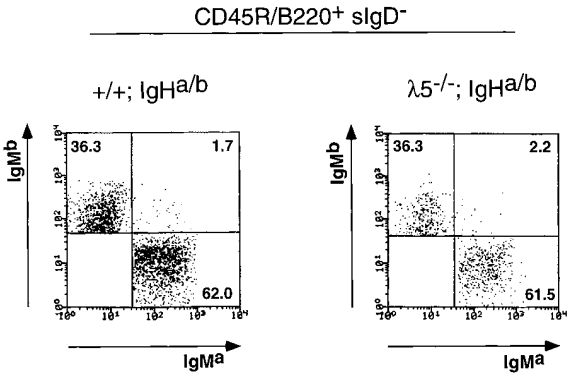

Figure 2. Newly Generated B Cells of λ5-Deficient Mice Are Allelically Excluded at the Level of Immunoglobulin Cell Surface Expression

Flow cytometric analysis of newly generated B cells from a 8-week-old F1(C57BL/6 × BALB/c) mouse (left) and a 4-week-old F1(λ5T/λ5T,IgH^a × λ5T/λ5T,IgH^b) mouse (right) for the presence of μ chain allotype double-producing cells.

As depicted in Figure 2, in λ5-deficient mice and in control animals only 2.2% and 1.7% of the cells, respectively, stained positive for both allotypes (varying from 1.6%–2.7% of $sIgM^+sIgD^-$ bone marrow B lymphocytes in different experiments; data not shown). To find out whether these few cells are indeed double producers, we examined the IgH gene rearrangements of four such cells by single-cell PCR. One cell carried a combination of a productive and a nonproductive $V_HD_HJ_H$ joint and each of the three other cells contained a productive $V_HD_HJ_H$ joint and a D_HJ_H rearrangement (data not shown). This result suggests that the observed double producers in the compartment of newly generated B cells represent a staining artifact.

We conclude that double-producing B progenitor cells in λ5-deficient mice are already eliminated before they express antibody molecules on the cell surface.

Dμ Proteins Mediate Allelic Exclusion in Early B Progenitor Cells in Conjunction with the Surrogate L Chain

In support of the model of Dμ protein–mediated allelic exclusion at the IgH locus, Ehlich et al. (1994) found a random distribution of D_H RFs in early B cell progenitors harboring two D_HJ_H rearranged alleles, whereas Dμ protein–encoding joints (RF2) were strongly underrepresented in cells that carried a D_HJ_H joint and a $V_HD_HJ_H$ rearrangement. This result contradicts the hypothesis of Haasner et al. (1994), which proposes that Dμ proteins prevent proliferation of the respective B progenitor cell that leads to outgrowth of mainly those B cell precursors that carry D_HJ_H joints in RF1 and RF3.

To investigate whether, as suggested, down-regulation of $V_H \rightarrow D_HJ_H$ joining by Dμ protein expression is dependent on the presence of the surrogate L chain, we compared the D_H element RF usage in IgH gene rearrangements in B lineage cells of various developmental stages between wild-type and λ5-deficient mice. For details, see Experimental Procedures.

Table 3. D_H Reading Frame Usage in D_HJ_H and $V_HD_HJ_H$ Rearrangements of $CD43^+$ and $CD43^-$ B Cell Progenitor Cells as well as of Splenic B Cells of Wild-Type and λ5-Deficient Mice

		Wild-type mice			λ5T/λ5T mice		
Cells	Rearrangement	RF1	RF2	RF3	RF1	RF2	RF3
$CD43^+$ B cell progenitors	DJ	9	9[b]	9	19 (1)	19	19
	VDJ^+	27	4	4	6	5	1
	VDJ^-	9	2[b]	11	8	6	7
$CD43^+$ $c\mu^+$ B cell progenitors[a]	VDJ^+	9 (3)	0	0	7	7	5
	VDJ^-	8	1	5	3	3	4
$CD43^-$ B lineage cells	DJ	12 (1)	1	8			
	VDJ^+	24 (1)	8	5		–	
	VDJ^-	5	2	7			
Splenic B cells	DJ	7	1	9	10 (1)	3	10
	VDJ^+	12 (1)	1	1	11 (3)	8	4
	VDJ^-	2	2	1	6 (5)	6	6
		6	0	6^+			
Splenic B cells[a]	VDJ^+		ND		2 (3)	5	1
	VDJ^-				2 (2)	2	4

Numbers in parenthesis represent D_HJ_H or $V_HD_HJ_H$ joints with sequence homology at the recombinatorial breakpoints. IgH joints utilizing RF2 are represented in bold. For each cellular fraction, productive and nonproductive $V_HD_HJ_H$ rearrangements were obtained from the same library. For details, see Experimental Procedures. The statistical analysis using the χ^2 test indicates a nonrandom RF usage in $V_HD_HJ_H$ joints of the $CD43^+$ progenitor compartment as well as in D_HJ_H and $V_HD_HJ_H$ rearrangements isolated from B lineage cells of other developmental stages due to the underrepresentation of RF2 in wild-type mice ($p < 0.05$; except for $V_HD_HJ_H^-$ rearrangements isolated from $CD43^-$ B lineage cells and unselected $V_HD_HJ_H^-$ joints from splenic B cells due to the low number of joints analyzed in these cases). A plus indicates nonproductive $V_HD_HJ_H$ rearrangements selectively isolated (see Experimental Procedures). A dash indicates not analyzed (λ5T mice lack $CD43^-$ pre-B cells).
[a] Rearrangements obtained in the single-cell analysis; data taken from Table 1.
[b] Rearrangements utilizing RF2, including one nonproductive joint caused by a stop codon at the D_H–J_H border.
ND, not done.

Dμ protein–encoding D_HJ_H joints (RF2) are readily detectable in cells of the $CD43^+$ B cell progenitor compartment in wild-type and in λ5-deficient mice (Table 3). In wild-type animals, 30% (8 of 27 D_HJ_H joints) of the D_HJ_H rearrangements in this cell population represent D_HJ_H joints in RF2, and in λ5-deficient mice, 33% (19 of 57 D_HJ_H joints) of such joints are found among all D_HJ_H rearrangements. In contrast with this finding, D_HJ_H joints utilizing RF2 are strongly underrepresented at all later stages in B cell development in both wild-type and λ5-deficient mice (Table 3).

Although D_HJ_H joints of $CD43^+$ B precursor cells are generated in all three RFs, we observed an underrepresentation of the Dμ protein encoding RF in both productive (4 of 35 $V_HD_HJ_H$ joints, 11%; 0 of 12 $V_HD_HJ_H$ joints in the single-cell analysis) and nonproductive $V_HD_HJ_H$ rearrangements in the same cell population in wild-type mice (1 of 21 $V_HD_HJ_H$ joints, 5%; a second joint utilizing RF2 is not scored in this context because of a stop codon at the D_H–J_H border preventing Dμ protein expression; 1 of 14 $V_HD_HJ_H$ joints in single cells, 7%; Table 3). Furthermore, only few $V_HD_HJ_H$ joints containing a D_H element in RF2 were observed in $CD43^-$ B lineage cells and splenic B cells (Table 3).

The observation that RF2 is underrepresented in $V_HD_HJ_H$ but not D_HJ_H joints at the same developmental stage leads us to the same conclusion as that drawn by Ehlich and coworkers (1994); namely, that those $CD43^+$ progenitor B cells that may express a Dμ protein do not proceed with $V_H \rightarrow D_HJ_H$ joining and are therefore arrested in their development.

In striking contrast with the RF usage in $V_HD_HJ_H$ rearrangements of wild-type mice, $V_H \rightarrow D_HJ_H$ recombination in λ5-deficient mice involves Dμ protein–encoding joints as frequently as D_HJ_H joints in RF1 and RF3: 5 of 12 productive and 6 of 21 nonproductive $V_HD_HJ_H$ rearrangements isolated from $CD43^+$ progenitor cells utilized RF2 (Table 3). Again, this RF distribution is paralleled by that found in the single-cell analysis with 37% of the productive (7 of 19 $V_HD_HJ_H$ joints) and 30% of the nonproductive (3 of 10 $V_HD_HJ_H$ joints) $V_HD_HJ_H$ rearrangements using RF2. Furthermore, in splenic B cells, 35% of the productive (8 of 23 productive $V_HD_HJ_H$ joints; 5 of 8 productive $V_HD_HJ_H$ joints in the single-cell analysis) and 33% of the nonproductive (6 of 18 nonproductive $V_HD_HJ_H$ joints; 2 of 8 nonproductive $V_HD_HJ_H$ joints in single cells; Table 3) $V_HD_HJ_H$ rearrangements used this RF. Cells carrying a productive $V_HD_HJ_H$ joint and a Dμ protein–encoding rearrangement seem to be underrepresented in the peripheral B cell compartment (Table 3), although the number of D_HJ_H joints analyzed in this case is relatively low. However, these cells are as frequently generated in the bone marrow as $CD43^+c\mu^+$ cells harboring D_HJ_H rearrangements in RF1 or RF3 (data not shown). The apparent counterselection of cells coexpressing μ and Dμ proteins could be due to an impaired ability of these cells to express regular antibody molecules because of the formation of μ/Dμ chain heterodimers.

From the finding that RF2 is underrepresented in $V_HD_HJ_H$ joints in wild-type but not in λ5-deficient mice, we conclude that Dμ protein–mediated down-regulation of $V_H \rightarrow D_HJ_H$ recombination requires λ5 gene expression.

Discussion

Evidence for the Ordered Model of IgH Gene Rearrangements: Allelic Exclusion Mediated by μ Chains and Dμ Proteins in Cells of the IgH Gene-Recombining Progenitor Population

How allelic exclusion is established at the IgH locus, either by selection against double producers or by preventing a second $V_HD_HJ_H$ rearrangement once an IgH allele is productively assembled, is still a matter of debate, since experimental work has not so far allowed a direct analysis of allelic exclusion at the developmental stage at which it is established (see Oancea and Shulman, 1993; Rajewsky et al. 1993; Wabl and Steinberg, 1993). As a special problem, μ chain transgenic mice, although they show greatly decreased levels of endogenous $V_HD_HJ_H$ rearrangements, often contain a substantial fraction of double-producing B cells coexpressing the transgene-encoded μ chain and endogenous μ chains (summarized by Cohn and Langman, 1990; Lam et al., 1993; Imanishi-Kari et al., 1993).

The ordered model of IgH gene rearrangements makes the critical prediction that H chain allelic exclusion should be established already in the earliest progenitor population that contains $V_HD_HJ_H$ rearrangements; namely, cells at the $CD43^+$ stage. This prediction had not been tested so far. Our previous finding that $CD43^-$ pre-B cells are allelically excluded (Ehlich et al., 1994), does not exclude the possibility that double-producing B cell progenitors are generated at an earlier developmental stage (i.e., that of the $CD43^+$ progenitors). Such early double producers could have a survival disadvantage or could be competed out by allelically excluded proliferating progenitor cells during further maturation.

The present study demonstrates that B cell precursors are already allelically excluded at the developmental stage at which IgH gene recombination occurs. The finding that in $CD43^+$ cytoplasmic μ^+ cells that carry two $V_HD_HJ_H$ rearrangements only one $V_HD_HJ_H$ joint is productive indicates that μ chain expression leads to down-regulation of $V_H\rightarrow D_HJ_H$ recombination in the respective progenitor cell. From a mechanistic point of view, the initial step of allelic exclusion might include the μ chain–dependent transcriptional or posttranscriptional control of the recombination activating genes *RAG1* and *RAG2*, whose gene products are crucial for the initiation of $V_HD_HJ_H$ recombination (Schatz et al., 1989; Oettinger et al., 1990; van Gent et al., 1995). Lin and Desiderio (1994) have shown that RAG2 protein accumulates during the G1 phase of the cell cycle, while it is decreased in S, G2, and M phase. Since it has been suggested that B progenitor cells undergo rapid proliferation once they have acquired a functional $V_HD_HJ_H$ joint (Karasuyama et al., 1993), the μ chain–dependent transition from G1 to S phase might lead to suppression of $V_H\rightarrow D_HJ_H$ recombination due to the degradation of RAG2 protein (Lin and Desiderio, 1995). In aggreement with this notion, Grawunder et al. (1995) recently found that in μ^+ large B progenitor cells *RAG1* and *RAG2* mRNA levels are down-regulated, in contrast with μ^- progenitor cells. However, the control of *RAG* gene expression by μ chains cannot be the only factor controlling allelic exclusion at the IgH locus, because expression of *RAG1* and *RAG2* genes is again up-regulated in μ^+ small pre-B cells that undergo L chain gene rearrangements (Grawunder et al., 1995).

In line with the model of μ chain–dependent regulation of $V_H\rightarrow D_HJ_H$ recombination, we provide further evidence that Dμ proteins similarily mediate allelic exclusion at the IgH locus as had been also indicated by experiments of Ehlich et al. (1994). The strong underrepresentation of the Dμ protein encoding RF2 in $V_HD_HJ_H$ joints but not D_HJ_H joints of B progenitor cells at the same developmental stage is most easily explained by assuming that Dμ protein expression, possible only from D_HJ_H rearrangements in RF2, leads to inhibition of $V_H\rightarrow D_HJ_H$ joining in that cell. Those cells could not generate a $V_HD_HJ_H$ joint that seems to be required for a $CD43^+$ B progenitor cell to enter the next developmental stage, the compartment of $CD43^-$ pre-B cells (Ehlich et al., 1993; Reichman-Fried et al., 1993; Young et al., 1994; Spanopoulou et al., 1994; Ehlich et al., 1994). In support of this view and in accord with the earlier data of Gu et al. (1991a), we show that $CD43^-$ pre-B cells essentially lack D_HJ_H joints utilizing RF2 (Table 3), suggesting that Dμ protein expressing $CD43^+$ progenitor B cells are blocked from further differentiation.

Thus, the present data indicate that in wild-type mice, allelic exclusion at the IgH locus is established as soon as B progenitor cells express a μ chain or a Dμ protein. This finding supports the view of the ordered model of IgH gene recombination that postulates a regulatory function of the μ chain, exerting an inhibitory effect on further $V_H\rightarrow D_HJ_H$ recombination.

Allelic Exclusion at the IgH Locus Requires Pre-B Cell Receptor Expression

Mutant mice lacking a component of the pre-B cell receptor (either functional μ chains in J_HT mice [Gu et al., 1993; Chen et al., 1993] and μMT mice [Kitamura et al., 1991] or the surrogate L chain molecule λ5 [Kitamura et al., 1992]) show a severe block in early B cell development, indicating that the pre-B cell receptor guides the transition of CD43-expressing B progenitor cells to the stage of $CD43^-$ pre-B cells (Ehlich et al., 1993).

Since the block in B cell development in λ5-deficient mice is not complete, one can analyze in these animals allelic exclusion at the IgH locus at various stages in B cell development in the absence of surrogate L chain expression. Using λ5-deficient mice, we were thus able to differentiate whether the membrane-bound μ chain as such or the pre-B cell receptor is essential for allelic exclusion at the IgH locus.

In contrast with our finding that early progenitor cells are allelically excluded in wild-type mice, 5 of 16 cytoplasmic μ^+ $CD43^+$ B progenitor cells with two completely rearranged IgH loci were found to be allelically included in the absence of λ5 gene expression (Table 2). Thus, $V_H\rightarrow D_HJ_H$ recombination can proceed in the presence of a μ chain, but is blocked once the μ chain is assembled into a pre-B cell receptor. By analogy, in Dμ protein–expressing B progenitor cells, allelic exclusion is abrogated in the absence of λ5 protein as shown by the frequent usage of the Dμ protein encoding RF in $V_HD_HJ_H$ joints at all stages in B cell development (Table 3).

Recently, Papavasiliou and colleagues (1995) demonstrated that $V_H \rightarrow D_H J_H$ joining proceeds in B cell progenitors of mice that express a transgene-encoded μ chain that does not associate with the Igα–Igβ heterodimer. In the wild type, these two polypeptides associate with the μ chain and are supposed to function in signal transduction via the B cell antigen receptor (reviewed by Cambier et al., 1994). This finding implies that allelic exclusion is connected to a signaling function of the μ chain. Our experiments provide evidence that this putative signaling function of the μ chain is mediated by a pre-B cell receptor (involving either a conventional μ chain or a Dμ chain), and supports the idea that these two pre-B cell receptors prevent the recombination machinery from further $V_H \rightarrow D_H J_H$ joining.

Why Do Allelically Included B Cell Progenitors Not Significantly Contribute to the Peripheral B Cell Pool in λ5-Deficient Mice?

Two interpretations offer themselves to explain the virtual absence of double producers in the B cell population of λ5-deficient mice despite their presence in the compartment of $CD43^+$ progenitors. Either such B cells are counterselected at the expense of allelically excluded B cells, or the double-producing progenitors do not or only rarely differentiate into B cells. We consider the former possibility unlikely for the following reasons. First, double-producing B cells can, in principle, survive in the lymphoid compartment of the mouse, as demonstrated in μ chain transgenic mice (reviewed by Cohn and Langman, 1990; Lam et al., 1993; Imanishi-Kari et al., 1993) and heterozygous μMT mice (Kitamura and Rajewsky, 1992). We have ourselves recently shown that gene-targeted mice expressing different $V_H D_H J_H$ rearrangements from their IgH alleles have normal or close to normal numbers of B cells in their peripheral B cell compartment, and the vast majority of these cells express both H chains (E. Sonoda, Y. Pewzner, D. Eilat, and K. R., unpublished data). Second, and most importantly, double producers are already undetectable in the compartment of newly generated B cells in the bone marrow of λ5-deficient animals (Figure 2).

We therefore believe that in the mutant animals the contribution of the allelically included progenitors to the B cell pool is negligible from the beginning onward. This could be due to a failure of these cells to rearrange their L chain gene loci efficiently, owing to the absence of an appropriate signal given by the pre-B cell receptor (Iglesias et al., 1991; Tsubata et al., 1992). B cell generation in the mutant animals would thus depend on the low level of L chain gene rearrangements that is known to occur independently of μ chain expression (Kitamura et al., 1992; Ehlich et al., 1993; Chen et al., 1993). Given a limited lifespan of the $CD43^+$ progenitors, this may favor the appearance of B cells that are derived from young progenitor cells, which did not yet perform $V_H \rightarrow D_H J_H$ rearrangements on both chromosomes. It has also been speculated that μ chain expression from both chromosomes may be toxic for the cell and may thus negatively interfere with further cellular differentiation (H chain toxicity; Köhler, 1980; Wabl and Steinberg, 1982). The apparent counterselection of the Dμ protein encoding RF2 in $D_H J_H$ (but not in $V_H D_H J_H$) rearrangements of B cells from λ5-deficient mice (Table 3) could be taken as support of this notion. However, this or any other mechanism of counterselection of allelically included B lineage cells would only apply to the λ5-deficient mouse mutant and perhaps evolutionary times before the invention of the pre-B cell receptor. In the wild type, double-producing cells are apparently not generated at any stage of progenitor development, most likely owing to the inhibition of $V_H \rightarrow D_H J_H$ joining by a signal delivered through the pre-B cell receptor.

Experimental Procedures

Cell Preparation

Single-cell suspensions from spleens of λ5T mice (Kitamura et al., 1992) and 129/Sv mice (bred in our animal facility) were prepared by crushing splenic tissue, and from bone marrow (femur) of C57BL/6 mice (Bomholtgaard, Denmark) and λ5T mice by flushing bones with medium (Dulbecco's modified Eagle's medium containing 5% fetal calf serum). Erythrocytes were removed from the preparations by lysis with Tris-buffered 0.165 M NH_4Cl. Bone marrow single-cell suspensions were washed by centrifugation through fetal calf serum. Mice were analyzed at the age of 8–14 weeks.

Flow Cytometric Analysis of Newly Generated B Cells

Bone marrow single-cell suspensions were prepared as described. Analysis of cells was performed by staining pooled cells with a combination of biotin-3.5-biotin (anti-δ; Roes et al., 1995), allophycocyanin–RA3.6B2 (anti-CD45R/B220), phycoerythrin–MB86 (anti-μ^b; Nishikawa et al., 1986), and fluorescein–RS3.1 (anti-μ^a; Schüppel et al., 1987), washing, and counterstaining with Texas red–avidin (Boehringer Mannheim). Cells were then analyzed using a dual laser-dye laser cytometer (FACStar plus, Becton-Dickinson). Lymphocytes were acquired through the lymphocyte gate by forward scatter and side scatter characteristics. Dead cells and cells displaying IgD on the cell surface were excluded from the analysis by staining with propidium iodine and anti-IgD antibody. Gating for newly generated B cells of homozygous λ5T mice was principally performed as the analysis of lymphocytes from F1 wild-type mice, except that lymphocytes were directly acquired as $CD45R/B220^+IgMa^+IgD^-$ and $CD45R/B220^+IgMb^+IgD^-$ lymphocytes because of the low numbers of such cells present in the bone marrow of these mutant mice.

Cell Sorting

Cells were isolated by fluorescence-activated cell sorting using a FACStar plus (Becton Dickinson). Dead cells were excluded from the analysis by staining with propidium iodide. Splenic B cells were sorted as $B220^+IgM^+$ cells using antibodies phycoerythrin–RA3.6B2 (anti-CD45R/B220) and fluorescein–R33-24-12 (anti-μ) or a combination of allophycocyanin–RA3.6B2, fluorescein–MB86 (Nishikawa et al., 1986), and phycoerythrin–RS3.1 (Schüppel et al., 1987). $CD43^+$ and $CD43^-$ B precursor fractions were sorted as described by Ehlich et al. (1994). B cell progenitor fractions are characterized by cell surface expression of markers CD43, CD45R/B220, HSA, and BP-1 (fraction A, $CD45R/B220^+CD43^+HSA^-BP\text{-}1^-$; fraction B, $CD45R/B220^+CD43^+HSA^+BP\text{-}1^-$; fraction C, $CD45R/B220^+CD43^+HSA^+BP\text{-}1^+$; fraction D, $CD45R/B220^+CD43^-sIgM^-$; fraction E, $CD45R/B220^+CD43^-sIgM^+sIgD^-$). Macrophages were removed by staining pooled bone marrow cells with M1/70.15.11-coupled (anti-MAC1; Springer et al., 1978) magnetic beads (Miltenyi Biotec), followed by magnetic cell sorting using a MACS system (Miltenyi et al., 1990) or during the cell sorting procedure by staining with fluorescein–M1/70.15.11. Sorted cells were washed by centrifugation in phosphate-buffered saline and stored as 5 μl aliquots (containing 1×10^4 to 2.5×10^4 cells) in 0.5 ml microtubes at −20°C.

To obtain cytoplasmic μ^+ cells from fraction C, we sorted 1×10^5 $B220^+CD43^+HSA^+BP\text{-}1^+$ cells from either C57BL/6 mice or λ5-deficient mice as described above. The gate for sorting cells of fraction C was set in a way that even when gating on $CD43^-$ pre-B cells, the latter cells should not enter the sorting gate. Theoretically,

Table 4. Correlation between the Numbers of PCR Products and Cells per Sample

Cells per sample	Number of samples	PCR products			
		B1-8 + T15	B1-8	T15	No product
1	97	0	37	39	21
2	85	27	20	29	9

even in the case of the highest possible contamination of $CD43^+$ pre-B cells by $CD43^-$ pre-B cells, at most 3 of 19 $c\mu^+$ cells analyzed could have been derived from fraction D (data not shown). However, such a contamination is unlikely, since the cells isolated appeared $CD43^+$ on the cell surface.

Sorted cells were fixed in 2% formaldehyde, phosphate-buffered saline (PBS) for 20 min at room temperature. After washing with PBS, cells were resuspended in PBS containing 1% bovine serum albumin and 0.1% NaN_3. Cells were bleached overnight and stained for cytoplasmic μ chains in the presence of 1% saponine (Sigma) using the anti-μ monoclonal antibody M41 (Leptin et al., 1984). After washing, single cytoplasmic μ^+ cells were deposited into 0.5 ml microtubes containing 20 μl PCR buffer (GIBCO BRL, 2.5 mM $MgCl_2$) supplemented with 1 μg/ml rRNA from Escherichia coli (Boehringer), frozen on dry ice, and stored at −80°C.

Isolation of D_HJ_H- and $V_HD_HJ_H$ Joints from B Cell Precursor Populations and Splenic B Cells

Cell samples were lysed prior to the PCR by adding 20 μl PCR buffer (10 mM Tris–HCl [pH 8.3], 50 mM KCl, 2.5 or 3.0 mM $MgCl_2$, 100 mg/ml gelatin), overlaying the sample with paraffin oil, and heating it for 10 min at 96°C. After digestion with proteinase K (0.5 mg/ml; Boehringer Mannheim) for 2 hr at 55°C, cell samples were heated again for 15 min to 96°C to inactivate proteinase K. PCR buffer (25 μl) containing 200 μM of each dNTP (final concentration; Pharmacia), 10–20 pmol of primers, and 5 U Taq–polymerase (GIBCO BRL) was added to the cell preparations. D_HJ_H and $V_HD_HJ_H$ joints were amplified from cell lysates containing 1–2.5 × 10^4 sorted cells using PCR performed for 35 cycles using a Techne thermal cycler or a Biometra thermal cycler. Each cycle consisted of 0.45 min at 94°C, 1.15 min at 68°C, and 1.30 min at 72°C. The primers used in the reactions have been described earlier: 5′ to D_H (Gu et al., 1991a), V_HJ558 genes (Gu et al., 1991b; used only for PCR of $V_HD_HJ_H$ rearrangements derived from splenic B cells), 3′ to J_H1 (Weiss and Rajewsky, 1990), and 3′ to J_H4 (Gu et al., 1991a). The primer specific for V_HJ558 genes used for amplification of $V_HD_HJ_H$ joints from sorted bone marrow B lineage cells was as follows: 5′ primer V_HJ558, 5′-GCGAAGCT-TA(AG)GCCTGGG(AG)CTTCAGTGAAG-3′. D_HJ_H rearrangements of $CD43^+$ B cell progenitors were isolated from cells of fractions A and B in wild-type mice and of fractions A–C in λ5T mice. D_HJ_H and $V_HD_HJ_H$ rearrangements of $CD43^-$ B lineage cells comprise joints isolated from cells of fraction D and E, since RF usage in both fractions did not differ significantly (data not shown).

To facilitate the analysis of nonproductive joints isolated from splenic B cells of 129/Sv mice, we screened selectively for nonproductive $V_HJ558D_HJ_H1$ joints by cloning the PCR products into the PstI–XbaI sites of the vector pTZ19(R) (Pharmacia), disrupting the encoded lacZ α–peptide gene. With the ligated vector DNA, transformed bacteria ($D_H5\alpha$) were plated on dyt plates containing the selection marker ampicilin and IPTG/X-Gal. Colonies containing productive $V_HD_HJ_H$ rearrangements showed β-galactosidase activity rendering the colonies blue, whereas colonies bearing a nonfunctional $V_HD_HJ_H$ joint cannot undergo lacZ α–peptide complementation and appeared white.

PCR Analysis of IgH Gene Rearrangements from Single Cells

To prepare genomic DNA for amplification, samples were overlaid with paraffin oil and incubated for 1 hr with proteinase K (0.5 mg/ml; Boehringer) at 55°C. Then, proteinase K was inactivated at 96°C for 10 min. IgH gene rearrangement amplification was carried out in two rounds: the first round contained all 5′ primers and the J_H4E primer (3 pmol each) as described by Ehlich et al. (1994) and, in addition, a 5′ primer hybridizing to members of the V_H8 gene family (GCGAAGCTTCCCTGGGATATTGCAGCCCTC). Amplification was done for 30 cycles (0.45 min at 94°C, 1 min at 60°C, 2.5 min at 72°C). For the second round of PCR, 1.5 μl aliquots of the first round reaction were transferred into seperate reactions, each containing a single primer combination of a V_H gene family-specific primer (or D_H-specific primer) and including a nested J_H4A primer. Mouse embryonic stem cells served as negative controls in the PCR. Amplifications from single cells that yielded more than two PCR products per cell were characterized by a third round of PCR and sequencing. In those cases, the additional PCR products were shown to be identical to the previously analyzed joints of the cell (due to cross-hybridization of primers) or represented PCR artifacts. In none of those cases were additional IgH rearrangements detectable, supporting the finding of the control experiment that only one cell was sorted into each microtube (see below).

Control Experiment to Confirm the Isolation of Single Cells by FACS

Two mutant mouse strains were chosen that carried rearranged IgH chain V region genes introduced by gene targeting into the H chain locus, replacing the J_H locus (T15i mice, Taki et al., 1993; B1-8i mice, E. Sonoda and K. R., unpublished data, containing a rearranged $V_H186.2$ gene isolated from the hybridoma B1-8; Bothwell et al., 1981). Of each of the two mouse strains homozygous for the introduced H chain, 1 × 10^5 $B220^+CD43^+$ bone marrow cells were sorted by FACS and mixed in a 1:1 ratio. Cells were fixed and stained for cytoplasmic μ chain expression as described above. Of these cells, single cells as well as doublets were resorted into 0.5 ml microtubes as previously described and $V_HD_HJ_H$ rearrangements were amplified in a seminested PCR approach using the following primers: V_HT15, GCGAAGCTT(AT)CTGGAGGAGGCTTGGTGCAG; J_H1E, ACGCTCTG AGATCCCGGGATCTGCAATATC; J_H1A, CCCGTTTCAGAATGGAAT GTGC; V_HB1-8, GCGAAGCTTA(AG)GCCTGGG(AG)CTTCAGTGAAG; J_H2E, GTGTCCCTAGTCCTTCATGAC; J_H2A, ATGCAGTAAAATCTAT CTAAGCTG.

The outcome of this experiment is shown in Table 4, confirming that every microtube that should contain a single cell indeed showed only amplification of either of the expected PCR products.

DNA Sequencing

PCR products were gel-purified and cloned into PTZ19(R) vector following digestion with the appropriate restriction enzymes. Bacterial colonies ($D_H5\alpha$) containing D_HJ_H or $V_HD_HJ_H$ rearrangements were randomly picked, and D_HJ_H as well as $V_HD_HJ_H$ sequences were determined by radioactive sequencing of double-stranded plasmid DNA following the protocol of the Sequenase kit (United States Biochemical). PCR products obtained from single-cell PCR were gel-purified from ethidium-stained 1.2% agarose gels using Quiaex II gel extraction kit (Qiagen). Cycle sequencing was performed using the Ready Reaction DyeDeoxyTerminator Cycle sequencing kit (Applied Biosystems) following the instructions of the manufacturer and sequenced by automated sequencing (Applied Biosystems). Sequencing primers recognizing sequences downstream of the respective J_H genes were as described by Ehlich et al. (1994).

Acknowledgments

We are grateful to G. Zoebelein and C. Göttlinger for technical help, U. Ringeisen for preparing the figures, and Dr. H. Kneser for discussion. This work was supported by the Deutsche Forschungsgemeinschaft through SFB 243, the Land Nordrhein-Westfalen, and the Human Frontier Science Program. D. Löffert was supported by a fellowship from the Fonds der Chemie.

Received October 26, 1995; revised December 26, 1996.

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GenBank Accession Numbers

The nucleotide sequence data of the IgH rearrangements reported in Table 3 will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession numbers X96001–X96371.