

# Restricted Immunoglobulin Variable Region (Ig V) Gene Expression Accompanies Secondary Rearrangements of Light Chain Ig V Genes in Mouse Plasmacytomas

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## Summary

The many binding studies of monoclonal immunoglobulin (Ig) produced by plasmacytomas have found no universally common binding properties, but instead, groups of plasmacytomas with specific antigen-binding activities to haptens such as phosphorylcholine, dextrans, fructofuranans, or dinitrophenyl. Subsequently, it was found that plasmacytomas with similar binding chain specificities not only expressed the same idiotype, but rearranged the same light ( $V_L$ ) and heavy ( $V_H$ ) variable region genes to express a characteristic monoclonal antibody. In this study, we have examined by enzyme-linked immunosorbent assay five antibodies secreted by silicone-induced mouse plasmacytomas using a broader panel of antigens including actin, myosin, tubulin, single-stranded DNA, and double-stranded DNA. We have determined the Ig heavy and light chain V gene usage in these same plasmacytomas at the DNA and RNA level. Our studies reveal: (a) antibodies secreted by plasmacytomas bind to different antigens in a manner similar to that observed for natural autoantibodies; (b) the expressed Ig heavy genes are restricted in V gene usage to the  $V_H$ -J558 family; and (c) secondary rearrangements occur at the light chain level with at least three plasmacytomas expressing both  $\kappa$  and  $\lambda$  light chain genes. These results suggest that plasmacytomas use a restricted population of B cells that may still be undergoing rearrangement, thereby bypassing the allelic exclusion normally associated with expression of antibody genes.

Key words: V(D)J rearrangement • plasmacytoma • allelic exclusion • polyreactivity • V gene usage

Pristane- and mineral oil-induced mouse plasmacytomas (PCs)<sup>1</sup> have proved to be invaluable in the study of Ab diversity, as well as in the chromosomal translocations associated with the development of late-stage B cell tumors. Virtually all mouse PCs exhibit a nonrandom chromosomal translocation between the Ig heavy chain or light chain gene and the *c-Myc/Pvt 1* gene locus (1). As the original source of homogeneous Abs, PCs exhibit allelic exclusion and express a single unique heavy chain and light chain Ig molecule. Although some PCs have demonstrated reactivity to specific Ags (i.e., DNP,  $\alpha$ 1,3- and  $\alpha$ 1,6-dextrans, phosphorylcholine, levan, and  $\beta$ 2,1- and  $\beta$ 2,6-fructofuranans), a single predominant Ag or hapten target has not sur-

passed despite nearly three decades of research (2). In earlier studies, we found that natural polyreactive autoantibodies (NAAs) are an important component of the normal B cell repertoire, and that hybridomas derived from the spleens of BALB/c mice frequently exhibited polyreactivity to a panel of Ags including actin, myosin, tubulin, single-stranded (ss)DNA, and double-stranded (ds)DNA (3–5). Although NAAs are of low affinity and are encoded by essentially germline V region sequences (6), pathogenic Abs implicated in autoimmune disease are monospecific with higher affinity, and exhibit Ag-driven somatic mutation. In addition, considerable data have accumulated in humans indicating that the autoreactive repertoire frequently undergoes malignant transformation. This evidence has accumulated in studies of chronic lymphocytic leukemia (CLL), follicular non-Hodgkin lymphomas (FNHLs), and monoclonal Ig (for review, see reference 7). Paradoxically, the NAA specificity frequently found among human monoclonal Ig has not yet been reported in mouse PCs. In this report, we

<sup>1</sup>Abbreviations used in this paper: CLL, chronic lymphocytic leukemia; ds, double-stranded; g, generation; GC, germinal center; IVS, intervening sequence; NAA, natural polyreactive autoantibody; PC, plasmacytoma; R, replacement; RAG, recombination activating gene; RT, reverse transcription; S, silent; SI, silicone-induced PC; ss, single-stranded.

have searched for and found polyreactivity to several Ags, including myosin, dsDNA, and ssDNA in BALB/c silicone-induced PCs (SIPCs) of primarily the IgA heavy chain class.

Since the analysis of V region nucleotide sequences can also provide insight into the stage of B cell development at which clonal expansion occurs, as well as the putative role that an Ag-driven process could play in the selection of malignant B cell tumors, we have examined and identified restricted V region usage in the SIPC tumors. We have also observed Ab diversification in the form of secondary rearrangements that may be targeting the B cell population (B-1) in the periphery, apparently in an attempt to diverge from the polyreactive response and possible autoimmunity.

## Materials and Methods

**Tumors.** SIPCs were generated by three successive inoculations (on days 0, 60, and 120) of silicone gel into the peritoneal cavity of BALB/c or BALB/c.DBA/2N-Idh1-Pep3 congenic mice (8). The latency times for first generation tumors were SIPC3301 (227 d), SIPC3308 (190 d), SIPC3282 (152 d), SIPC3336 (220 d) and SIPC3385 (225 d). Tumors were transplanted either with or without priming into syngeneic mice, and several generations (g) were examined, including: SIPC3301 (g1, g2), SIPC3308 (g0, g1, g2), SIPC3282 (g0, g1, g3), SIPC3336 (g1, g2, g3, g4), and SIPC3385 (g0, g2, g3).

**ELISA Assay.** SIPCs were screened for Ig secretion and Ab activity against actin, myosin, tubulin, dsDNA, and ssDNA (5). In brief, polystyrene flat-bottomed plates were coated with different Ags. After incubation with serial dilutions of each sample in duplicate, peroxidase-conjugated anti-mouse Ig was added; dilution medium alone was included as the negative control. Each assay was done in triplicate.

**Southern Hybridization and Slot Blot Analysis.** Approximately 10  $\mu$ g of EcoR1- or BamH1-digested genomic DNA was size separated on 0.7% agarose gels. After electrophoresis, the gels were acid (HCl) treated, alkaline treated, and neutralized in 3 M NaCl, 0.5 M Tris-Cl, pH 7.6. Southern transfers were made to Nytran Plus filters (Schleicher and Schuell), UV cross-linked, and hybridized to  $^{32}$ P-labeled DNA probes at 65°C with a final wash stringency of 0.2 $\times$  SSC at 65°C. The probes used to detect light chain  $\kappa$  rearrangements were either the 1.1-kb HindIII-XbaI (intervening sequence [IVS]) or the 1.8-kb HindIII-XbaI ( $J_{\kappa}$ ) fragments. For heavy chain Ig rearrangements, a 0.8-kb EcoR1  $J_H$  probe was used. Translocation to *c-Myc* or *Pvt 1* was assayed by screening EcoR1- or BamH1-digested filters with a 1.7-kb exon 2 PstI (*c-Myc*) or a 1.7-kb cDNA (*Pvt 1*) probe.

For slot blot hybridizations,  $\sim$ 1  $\mu$ g of RNAs from BALB/c.DBA/2N congenic mouse liver and spleen, as well as from each of the SIPC tumors used in this study, were applied to Hybond (Nycomed Amersham plc) filters and hybridized to *Ly1*, recombination activating gene 1 (*RAG-1*) cDNA,  $V_{\lambda}$ ,  $V_{\kappa}$ -24,  $V_{\kappa}$ -1, or  $C_{\kappa}$ -specific probes. After hybridization, the filters were washed at 0.2 $\times$  SSC at 65°C for 30 min.

**Spectral Karyotyping (SKY<sup>TM</sup>) of SIPC Tumors.** Although detailed methods have been reported elsewhere (9), metaphase cells were equilibrated in 2 $\times$  SSC, digested with RNase A and pepsin, fixed in 1% formaldehyde, denatured in 70% formamide/2 $\times$  SSC, and then dried. The fluorochromes, spectrum orange (dUTP conjugate; Vysis), rhodamine 110 (Perkin-Elmer), and

Texas red (12-dUTP conjugate; Molecular Probes) were used for direct labeling, and the haptens biotin-16-dUTP and digoxigenin-11-dUTP (Boehringer Mannheim Corp.) were used for indirect labeling. The probes were precipitated in an excess of mouse DNA (COT-1 DNA; GIBCO BRL), and hybridized at 37°C for 72 h in 50% formamide/SSC/dextran sulfate. After hybridization and washing (4 $\times$  SSC/Tween 20), the biotin was visualized by avidin-Cy5 (Nycomed Amersham plc), and the digoxigenin-11-dUTP was visualized by mouse antidigoxigenin (Sigma Chemical Co.) followed by sheep anti-mouse Cy5.5 (Nycomed Amersham plc). Chromosomes were counterstained with 4,6-diamino-2-phenylindole (DAPI) and embedded in antifade solution (1,4-phenylenediamine, Sigma Chemical Co.). Spectral images were obtained on a Leica DMRBE epifluorescence microscope equipped with an SD200 SpectraCube<sup>®</sup> (Applied Spectral Imaging) and a customized triple bandpass optical filter. Spectrum-based classification of the raw spectral images was performed using the software SkyView (Applied Spectral Imaging).

**Cloning and Subcloning of Rearranged V(D)J Genes.** Approximately 100  $\mu$ g of genomic DNA from SIPC3336 was restricted with EcoR1 and subjected to low-melting (1.0%) gel electrophoresis. Bands corresponding to the rearranged V(D)J genes were excised, extracted with phenol, and precipitated with ethanol. Inserts were then ligated to either EMBL4 or  $\lambda$ gt10 arms (EcoR1 digested). After ligation, the phage DNA was in vitro packaged and plated to densities of 125,000 per 20  $\times$  20 cm LB plate. Benton-Davis transfer of the phage to nitrocellulose filters was followed by hybridization to  $^{32}$ P-labeled IVS or  $J_H$  probes. Positive phage were mapped by restriction endonucleases, and inserts were subcloned into pGEM-4Z.

**PCR Amplifications and Sequencing of Heavy and Light Chain Ig.** Total cellular RNA was extracted from tumor tissues by RNA PLUS solution (Bioprobe Systems), and first-strand synthesis was carried out as described previously (5). PCR amplifications were set up in 50- $\mu$ l volumes containing 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 mM of each dNTP, 2 U of Taq DNA polymerase (GIBCO BRL), 50 pmol of each primer, and a 3  $\mu$ l aliquot of the cDNA reaction. The primer sequences used to amplify specific heavy chain or light chain V regions are shown in Table I. The primers for amplification of *Ly1*, *RAG-1*, and *RAG-2* are as follows: CCTAGATATCCAGGTGGT (*Ly1* 5'), GTGTCCTGGCACCAGCTG (*Ly1* 3'), AGGATCTGCA-TTCTCAGA (*RAG-1* 5'), TCAGCTTGCCTGTGCTCA (*RAG-1* 3'), GTCTCCAACAGTCAGACA (*RAG-2* 5'), and GCTCTTGCTATCTGTACA (*RAG-2* 3').

Each PCR amplification product was purified by agarose gel electrophoresis, extracted, and then subcloned into the TOPO-TA vector following the manufacturer's protocol (Invitrogen). Single bacterial colonies were isolated for direct sequencing. Sequencing was carried out according to the manufacturer's protocol (Promega). The nucleotide sequence data were analyzed, and comparisons were made with MacVector or Genetics Computer Group (GCG) software packages.

## Results

**SIPC Tumors Display Polyreactivity.** The ascites fluid of 33 different SIPC tumors was assessed for binding activity against actin, myosin, tubulin, ssDNA, and dsDNA by ELISA (10). We have focused on purified Abs from five of these tumors, each of which binds to at least one of the Ags

**Table I.** Primer Sequences Used in V Region-specific PCR Amplifications

V region primers	
V <sub>H</sub> -SG1	GTGCAGCTKMAGSAGTCRGG
V <sub>H</sub> -SG2	CARCTGCARCARYCTGG
V <sub>H</sub> -SG3	GTGAAGCTKSWGARTCTGG
V <sub>H</sub> -SG4	GTTCARCTKARCAGTCTGG
V <sub>H</sub> 13-3-5'	GCTGAACTGGCAAACCT
V <sub>H</sub> 13-3-3'	GTAATAGACTGCAGAGTC
V <sub>κ</sub> 1-FW1	AGTCTTGGAGATCAAGCC
V <sub>κ</sub> 24-CDR1	TCCTGCAGGTCTAGTAAG
V <sub>κ</sub> 24-FW3	GCCTCAGGAGTCCCAGAC
V <sub>κ</sub> 1,2	ATGTTGTGATGACCCA
V <sub>κ</sub> 4,5	AAATTGTTCTCACCCA
V <sub>κ</sub> 8,14,15,19,21	ACATTGTGMTGACHCA
V <sub>κ</sub> 9,12,13,31, 33,34	ACATCCAGATGACHCA
V <sub>κ</sub> 20	AAACAACCTGTGACCCA
V <sub>κ</sub> 23	ATATTGTGCTAACTCA
V <sub>κ</sub> 24,28	RTATTGTGATGACYCA
V <sub>λ</sub>	TTCCCAGGCTGTTGTGA
C region primers	
J <sub>H</sub> 3	GCAGAGAATCTTGGTCCT
IgG3	GTCACTGCAGCCAGGGACCA
IgA	GATGGTGGGATTTCTCGCACTC
IgGX	CAGGGGCCAGTGGATAGAC
J <sub>κ</sub> 2	CCTTAACACTTGATCTGA
J <sub>κ</sub> 4	CTATTGATGCACAGGTTG
C <sub>κ</sub>	ACACTCATTCCTGTTGAA
C <sub>λ</sub>	GAGCTCTTCAGAGGAAGG

from the panel. As far as heavy chain class, four out of the five SIPC3s examined (SIPC3308, SIPC3301, SIPC3336, and SIPC3282) in this study expressed IgA (only SIPC3385 expressed IgG3). Although most Abs show higher reactivity to myosin, several are particularly reactive to dsDNA and ssDNA. The tumor SIPC3301 displays low reactivity with essentially all Ags on this panel. The SIPC3282, SIPC3308, SIPC3336, and SIPC3385 Abs were found to display a polyreactive binding activity (Table II).

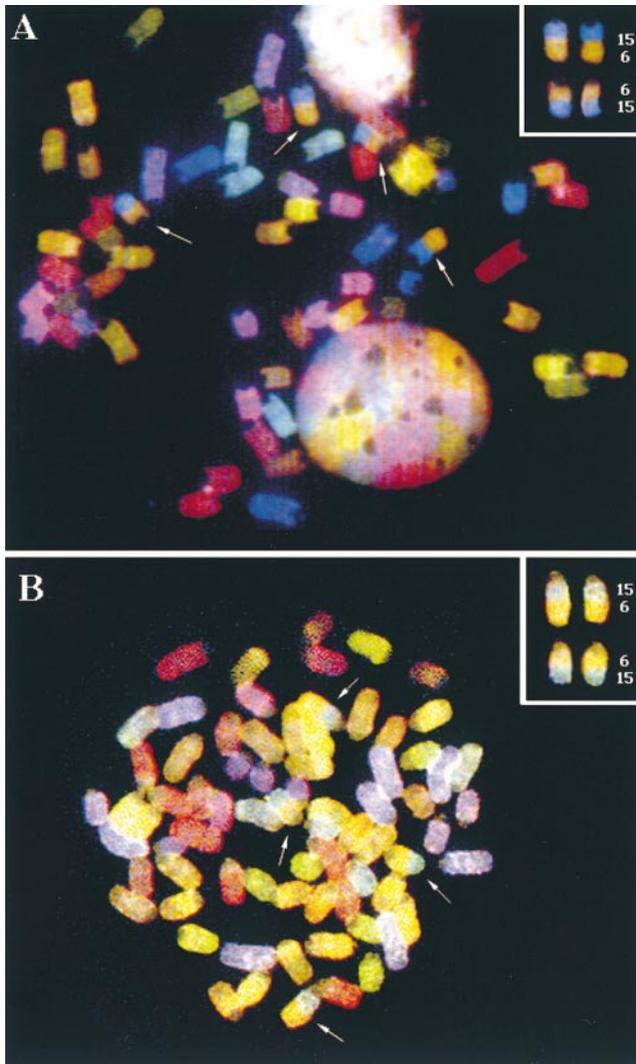
**Cytogenetics of SIPC Tumors.** Cytogenetic studies have revealed that a majority of PCs contain reciprocal T(12;15) translocations (1). Alternatively, several SIPC tumors exhibit a reciprocal T(6;15) translocation (considered to be the variant translocation), as demonstrated by the SKY™ analysis performed on two representative SIPC tumors (Fig. 1). While SKY™ cytogenetics is a good indicator of T(12;15) or T(6;15) translocations, it is not entirely certain whether *c-Myc* or *Pvt 1* is targeted by these translocations, as we have only found two SIPC tumors with *c-Myc* rearrangements (SIPC3385, SIPC3301) and no tumors with *Pvt 1* rearrangements at the Southern hybridization level (Table III, and Fig. 2). The assay for molecular rearrangement is based on utilization of a series of hybridization probes surrounding the major breakpoints of *Pvt 1* or *c-Myc*, and by incorporating large restriction fragments into the analyses (11). The absence of detectable rearrangements with *Pvt 1* or *c-Myc* in such a large number of SIPC tumors (Table III, and data not shown) suggests that, in general, SIPC-associated translocations could reside outside the usual or more common breakpoint locations, and may not be detectable with the probes used in this study.

**Ig V<sub>H</sub> Gene Expression.** Initially, Ig heavy chain-specific rearrangements were identified in each tumor by Southern blot analysis using a J<sub>H</sub> probe (Fig. 3). Heavy chain-specific rearrangements were found in all tumors, including apparent rearrangements of both alleles in SIPC3385, SIPC3282, and SIPC3301. We also found shared rearrangements with both BamH1 (not shown) and EcoR1 digestions in SIPC3308, SIPC3336, and SIPC3282, suggesting the same V<sub>H</sub> gene may be expressed in these tumors. We cloned and sequenced the 3.5-kb EcoR1 fragment from SIPC3336, and established that the rearrangement consists of a mem-

**Table II.** Reactivity Pattern of SIPC-purified Abs against Various Antigens

Abs	Isotype	Antigen activity as measured by ELISA*				
		Actin	Myosin	Tubulin	dsDNA	ssDNA
SIPC3385	IgG3	390 ± 19.519	845 ± 31	869 ± 12.529	1,632 ± 368.501	1,835 ± 1
SIPC3308	IgA	362 ± 1.732	1,178 ± 3.785	145 ± 17	287 ± 8.544	638 ± 8.504
SIPC3301	IgA	219 ± 41.501	250 ± 31	56 ± 7.549	188 ± 5	240 ± 34
SIPC3336	IgA	143 ± 1	1,253 ± 4	60 ± 2.645	126 ± 0	195 ± 12
SIPC3282	IgA	924 ± 1	1,224 ± 2.081	454 ± 145.502	1,505 ± 64	1,978 ± 81

\* Antigen-coated plates were incubated with purified Abs (1 μg/ml) or dilution medium, and then were incubated with a peroxidase anti-mouse IgA or IgG3 conjugate. The enzyme activity was detected at 450 nm, and the results are expressed in optical densities. Results are the average of triplicates ± SD.



**Figure 1.** SKY™ images of SIPC tumors with variant T(6;15) chromosomal translocations, (A) SIPC3308 and (B) SIPC3282. The arrows mark chromosomal translocations that have occurred between CHR6 (yellow) and CHR15 (blue). There are two copies of each T(6;15) and the reciprocal T(15;6).

ber of the  $V_H$ -J558 family (H13-3; reference 12) rearranged to  $D_H$ SP2-9 and  $J_H$ 3. To more specifically determine  $V_H$  gene usage in the SIPC tumors, we examined the expressed sequences by reverse transcription (RT)-PCR (Fig. 4). Indeed, three of the SIPC tumors (SIPC3282, SIPC3336, and SIPC3308) that share the 3.5-kb EcoR1 rearrangement all use the same H13-3 gene from the  $V_H$ -J558 family. We verified that this sequence was germline (nonmutated) by sequencing eight clones derived from the PCR product of BALB/c genomic DNA which was amplified by primer pairs specific for H13-3 (see Table I). Interestingly, these same three tumors have also rearranged to  $J_H$ 3, and use the same  $D_H$  region ( $D_H$ SP2-9) encoding the amino acid residues Trp-Phe. Although rearranged to  $J_H$ 3 and  $D_H$ SP2-9 as well, SIPC3301 has a longer nucleotide addition sequence, and is in fact encoded by another mem-

**Table III.** Cytogenetics of SIPC Tumors

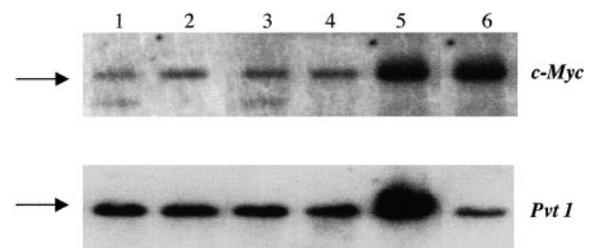
Tumors	SKY™ analysis	Molecular rearrangement*
SIPC3385	T(12;15)	<i>c-Myc</i> (18 kb)
SIPC3308	T(6;15)	NR†
SIPC3301	T(12;15)	<i>c-Myc</i> (18 kb)
SIPC3336	T(6;15)	NR
SIPC3282	T(6;15)	NR

\* Assayed by digestion with EcoR1 (*c-Myc*) or BamH1 (*Pvt 1*).

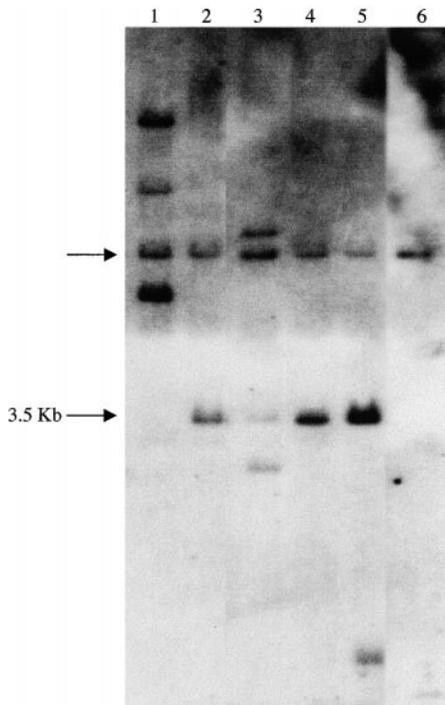
† No rearrangement (NR) with either *c-Myc* or *Pvt 1*.

ber (26.4.1- $\alpha$ ; reference 13) of the  $V_H$ -J558 family. The RT-PCR product of SIPC3385 was also found to use a member of the  $V_H$ -J558 family, but in this case the nitrophenyl-binding 4m3  $V_H$  gene (14). In SIPC3385, the rearrangement involves  $J_H$ 4 and another member of the  $D_H$ SP2 family with N sequence additions. Analysis of the  $V_H$  region sequences reveals somatic mutational activity with high replacement to silent (R/S) ratios, since all the SIPCs that express H13-3 exhibit four to six replacement changes with no accompanying silent base changes (Fig. 4). In the case of SIPC3385, we only find a single replacement in  $V_H$ .

**Ig  $V_L$  Gene Expression.** We examined the light chain-specific rearrangements of the SIPC tumors at the Southern hybridization level. The tumors SIPC3308, SIPC3336, and SIPC3282 all shared identical rearrangements with both EcoR1 (Fig. 5) and BamH1 (data not shown) digestions. By cloning and sequencing the 18-kb EcoR1 fragment (from SIPC3336), we obtained a  $V_{\kappa}$ 24C- $J_{\kappa}$ 4 (15) sequence suggesting that SIPC3308, SIPC3336, and SIPC3282 may all share the same rearrangement. When we performed PCR amplifications in each of these tumors with primers specific for  $V_{\kappa}$ 24C and  $J_{\kappa}$ 4, we obtained positive products that were identical as well as nonmutated (Fig. 6). To test whether the  $V_{\kappa}$ 24C- $J_{\kappa}$ 4 rearrangement is productive (expressed), we used  $V_{\kappa}$ 24C and  $C_{\kappa}$ -specific primers in RT-PCR assays, and again, found positive products in each of



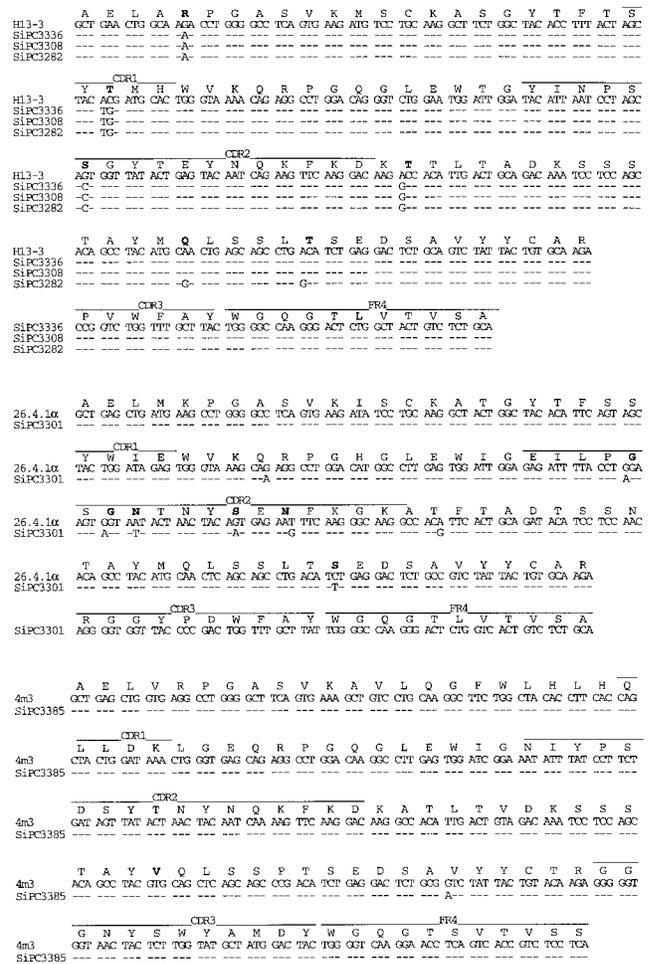
**Figure 2.** Southern hybridization assay for *c-Myc* and *Pvt 1* rearrangements in SIPC tumors. Shown top (*c-Myc*, EcoR1) and bottom (*Pvt 1*, BamH1) are the following tumors: lane 1, SIPC3385; lane 2, SIPC3308; lane 3, SIPC3301; lane 4, SIPC3336; lane 5, SIPC3282; and lane 6, BALB/c. Arrows depict the germline (nonrearranged) fragments. Two samples (SIPC3385 and SIPC3301) contain rearranged bands with *c-Myc*, whereas no rearrangements are found with *Pvt 1*.



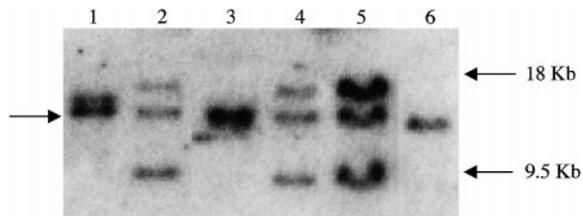
**Figure 3.**  $J_H$  rearrangements in SIPC tumors. Genomic DNAs from SIPC tumors and BALB/c liver were digested with EcoR1 and hybridized with the  $J_H$  probe: lane 1, SIPC3385; lane 2, SIPC3308; lane 3, SIPC3301; lane 4, SIPC3336; lane 5, SIPC3282; and lane 6, BALB/c. An arrow (left) depicts the germline (nonrearranged)  $J_H$  fragment, whereas the 3.5-kb EcoR1 rearrangement seen in several tumors is also highlighted.

these tumors (data not shown). Although the nature of the common 9.5-kb EcoR1 band found in SIPC3308, SIPC3336, and SIPC3282 is uncertain at the moment, we do know it must be related to the rearrangement to  $V_{\kappa}24C-J_{\kappa}4$ , since they are always found in association with the productive rearrangement in each of these tumors. Southern blots with a  $C_{\kappa}$  only (minus IVS) probe result in no hybridization to the 9.5-kb EcoR1 band, proving this fragment to be nonproductive and probably a byproduct of the rearrangement process (data not shown). Both SIPC3385 and SIPC3301 exhibit different rearrangements at the Southern blot level. Therefore, we performed RT-PCR amplifications to determine the expressed  $V_{\kappa}$  gene for these two tumors as well. The tumor SIPC3385 was found to express a nonmutated  $V_{\kappa}21G-J_{\kappa}2$  sequence (16), whereas SIPC3301 expressed a nonmutated  $V_{\kappa}34C-J_{\kappa}2$  sequence (17).

In the process of verifying the expressed  $V_{\kappa}$  sequences discussed above, we subjected all the SIPC tumors to RT-PCR amplification using sets of primers (Table I) that were cross-reactive to each of the  $V_{\kappa}$  and  $V_{\lambda}$  gene families. Unexpectedly, we found that in addition to expression of  $V_{\kappa}24C-J_{\kappa}4$ ,  $V_{\kappa}21G-J_{\kappa}2$ , and  $V_{\kappa}34C-J_{\kappa}2$ , each of the SIPC tumors expressed an additional  $Ig_{\kappa}$  or  $Ig_{\lambda}$  light chain gene, a finding in violation of allelic exclusion normally associated with Ab gene expression (18–20). All the SIPC tumors examined in this study were found to express either  $V_{\kappa}1A$  or  $V_{\kappa}1C$  (21, 22) rearranged to either  $J_{\kappa}1$ ,  $J_{\kappa}2$ , or  $J_{\kappa}4$  segments



**Figure 4.** Nucleotide sequences of  $V_H$  genes from SIPC tumors. The sequences obtained with the five SIPC tumors have been compared with the most homologous germline genes which are members of the  $V_H$ -J558 family. Top: SIPC3336, SIPC3308, and SIPC3282 express  $V_H13-3$ ; middle: SIPC3301 expresses 26.4.1- $\alpha$ ; bottom: SIPC3385 expresses 4ma. Dashes indicate sequence identities. Germline amino acids in bold indicate that they are replaced in SIPCs. These sequence data are available from EMBL/GenBank/DBJ under accession nos. AF154880–AF154882, AF154909, and AF154910.



**Figure 5.**  $Ig_L$  rearrangements of SIPC tumors. Genomic DNAs from SIPC tumors and BALB/c liver were digested with EcoR1 and hybridized with the IVS probe: lane 1, SIPC3385; lane 2, SIPC3308; lane 3, SIPC3301; lane 4, SIPC3336; lane 5, SIPC3282; and lane 6, BALB/c. An arrow (left) depicts the germline (nonrearranged)  $Ig_L$  fragment, whereas the 18 and 9.5-kb EcoR1 rearrangements seen in SIPC3308, SIPC3336, and SIPC3282 are also highlighted (right).



	CDR1																							
	T	L	T	C	R	S	S	T	G	A	V	T	A	T	S	N	Y	A	N	W	A	Q	E	K
S1PC3282B	ACA	CTC	ACT	TGT	CGC	TCA	AGT	ACT	GGG	GCT	GTT	ACA	ACT	AGT	AAC	TAT	GCC	AAC	TGG	GCT	CAA	GAA	AAA	
S1PC3282C	---	---	---	---	---	---	---	-T-	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
S1PC3282D	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
S1PC3282E	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
S1PC3282F	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
S1PC3336A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
S1PC3336B	---	---	---	---	---	---	---	-T-	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
S1PC3336C	---	---	---	---	---	---	---	---	---	---	C-	---	---	---	---	---	---	---	---	---	---	---	---	
S1PC3301A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
S1PC3301C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	

	CDR2																						
	P	D	H	L	F	T	G	L	I	G	G	T	N	N	R	A	P	G	V	P	A	R	F
S1PC3282B	CCA	GAT	CAT	TTA	TTC	ACT	GGT	CTA	ATA	GGT	GGT	ACC	AAC	AAC	CGA	GCT	CCA	GGT	GTT	CCT	GCC	AGA	TTC
S1PC3282C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
S1PC3282D	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-T-	---	---	---	---	---	---	---
S1PC3282E	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-T-	---	---	---	---	---	---	---
S1PC3282F	---	---	---	C-	---	---	---	---	---	---	---	---	---	---	---	-T-	---	---	---	A-	T-	---	---
S1PC3336A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
S1PC3336B	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	A-	---
S1PC3336C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	A-	---
S1PC3301A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	A-	---
S1PC3301C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	A-	---

	CDR3																						
	S	G	S	L	I	G	G	K	A	A	L	T	I	T	G	A	Q	T	E	D	E	A	I
S1PC3282B	TCA	GGC	TCC	CTG	ATT	GGA	GGC	AAG	GCT	GCC	CTC	ACC	ATC	ACA	GGG	GCA	CAG	ACT	GAG	GAT	GAG	GCA	ATA
S1PC3282C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
S1PC3282D	---	---	---	---	---	---	-A-	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
S1PC3282E	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
S1PC3282F	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
S1PC3336A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
S1PC3336B	---	---	---	---	---	---	-A-	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
S1PC3336C	---	---	---	---	---	---	-A-	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
S1PC3301A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
S1PC3301C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

	CDR3									
	Y	F	C	A	L	W	Y	S	N	H
S1PC3282B	TAT	TTC	TGT	GCT	CTA	TGG	TAC	AGC	AAC	CAT
S1PC3282C	---	---	---	---	---	---	---	---	---	---
S1PC3282D	---	---	---	---	---	---	---	---	---	---
S1PC3282E	---	---	---	---	---	---	---	---	---	---
S1PC3282F	---	---	---	---	---	---	---	---	---	---
S1PC3336A	---	---	---	---	---	---	---	---	---	---
S1PC3336B	---	---	---	---	---	---	---	---	---	---
S1PC3336C	---	---	---	---	---	---	---	---	---	---
S1PC3301A	---	---	---	---	---	---	---	---	---	---
S1PC3301C	---	---	---	---	---	---	---	---	TTC	TTC

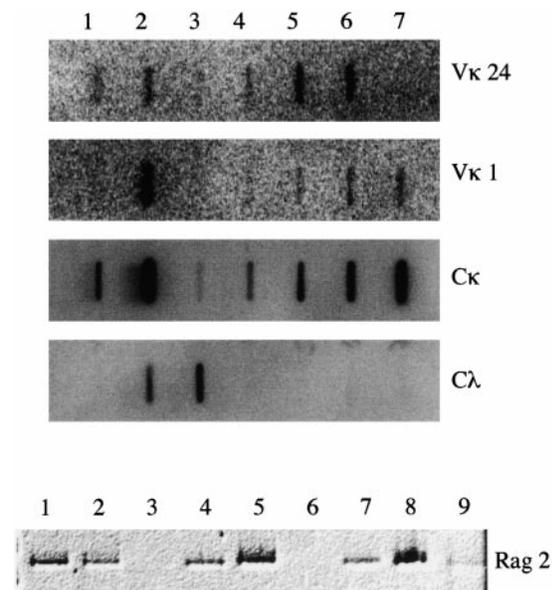
**Figure 8.** Nucleotide sequences of  $V_{\lambda 1}$  genes expressed in SIPC tumors. Nucleotide sequences of subclones (designated A–F) of  $V_{\lambda}$  genes from SIPC3282, SIPC3336, and SIPC3301 are compared with the most homologous germline gene. Dashes indicate sequence identities, and amino acid substitutions are indicated in bold. These sequence data are available from EMBL/GenBank/DDBJ under accession nos. AF154899–AF154908.

and  $V_{\kappa 24}$ . Since there is evidence that peritoneal cavity B cells are enriched in B-1 cells, we also tested for Ly1 expression by RT-PCR amplification of total RNA from thymus, spleen, two PCs, (ABPC18, MOPC104E) and the SIPC tumors. Although expression of Ly1 was found in thymus, spleen, and the two conventional PCs, no expression of Ly1 was evident in the SIPC tumors (data not shown). RAG-1 (data not shown) and RAG-2 (Fig. 9) activity, both of which have recently been found in the peripheral B-1 population (23), were also independently assayed by RT-PCR amplification among similar panels of RNAs. Both RAG-1 and RAG-2 expression were found in thymus, spleen, SIPC3301, SIPC3308, SIPC3385, ABPC18, and MOPC104E, but not in SIPC3282 or SIPC3336.

## Discussion

Since spontaneous PCs occur rarely in mice, studies in plasmacytomagenesis rely on induction models. All PCs arise in the peritoneum, where the presence of nonmetabolizable paraffin oils (pristane) or plastic implants induces chronic inflammation, granuloma formation, and finally development of the PC (1). The SIPC may differ by having fewer numbers of atypical foci (8). Binding studies of pristane-induced PCs showed that PCs can display binding activities against phosphorylcholine, various dextrans, and fructofuranans (2). The SIPCs, as we show in this study, possess a set of binding specificities against cytoskeletal proteins and DNA. In fact, in the initial panel of ascites from 33 SIPC tumors, binding activity against at least 1 of the Ags of the panel, and most often polyreactive binding activity, is commonly observed. To ascertain whether the SIPC tumors possess a characteristic set

of binding properties and whether these binding specificities could assist in identifying a precursor cell population, we focused on the binding activity of purified proteins from five representative SIPC tumors.



**Figure 9.** Transcript levels of  $Ig_{\kappa}$ ,  $Ig_{\lambda}$ , and RAG-2 in SIPC tumors. (Top) Equal amounts of RNA from mouse liver (lane 1), spleen (lane 2), SIPC3301 (lane 3), SIPC3308 (lane 4), SIPC3282 (lane 5), SIPC3336 (lane 6), and SIPC3385 (lane 7) were loaded into slots and hybridized to nick-translated probes from  $V_{\kappa 24}$  (row 1),  $V_{\kappa 1}$  (row 2),  $C_{\kappa}$  (row 3), or  $V_{\lambda}$  (row 4). (Bottom) RNA from mouse spleen (lane 1), thymus (lane 2), SIPC3282 (lane 3), SIPC3301 (lane 4), SIPC3308 (lane 5), SIPC3336 (lane 6), SIPC3385 (lane 7), ABPC18 (lane 8), and MOPC104E (lane 9) was reverse transcribed (RT) and PCR amplified using primers specific for RAG-2.

We have found that three independent SIPC tumors, each derived from different generations (SIPC3282, SIPC3308, and SIPC3336), share identical  $V_H$  domains including  $D_H$  and  $J_H$  regions (Table IV). Several facts argue strongly in favor of the independent derivation of these tumors: (a) each tumor was harvested at different times as a result of variable latency periods (see Materials and Methods); (b) reactivity patterns differ between each tumor (Table II); (c) SIPC3282 shows an additional rearrangement ( $J_H$ ) not found in other tumors, as well as additional somatic mutations not found in other tumors; (d) SIPC3385 and SIPC3308 both lack  $V_{\lambda}1$  rearrangements; and (e) no RAG-1/2 expression is found in SIPC3282 or SIPC3336. These results also support the existence of a strong restriction in V gene usage for the SIPCs. These results are in contrast with reports for human myeloma, where no particular selection for V genes has been observed (24). In contrast, Waldenström macroglobulinemia patients exhibiting rheumatoid or cold agglutinin anti-I specificities exhibit a strong restriction for V genes, as the V1-69  $V_{H1}$  gene member is almost constantly expressed in the case of cryoglobulins expressing the WA recurrent idio-type (60% of cases), and the V4-34 ( $V_{H4-21}$ ) gene is constantly expressed by cold agglutinins with anti-I specificity (95% of cases; [25]). Although the  $D_H$  region is not identical among these Abs, a report by Fais et al. (26) shows that among 50% of CLL patients expressing the 1-69 gene, the  $D_{H3-3}$  region is used. In addition, a report from the same group indicated that five different cases of  $CD5^+IgG^+$  CLLs expressed virtually identical Ag receptors, by recombining (unmutated) the  $V_{H4-39}$  gene to D6-13- $J_{H5b}$  and the  $V_{\kappa}012$  gene to  $J_{\kappa}1$  (27).

**Table IV.** Heavy and Light Chain Gene Usage in SIPC Tumors

Tumors	Expressed heavy chain	Expressed light chain		
		1	2	3
SIPC3385	4m3-DSP2- $J_H4^*$	$V_{\kappa}21G-J_{\kappa}2$	$V_{\kappa}1A-J_{\kappa}1$ $V_{\kappa}1C-J_{\kappa}2$	
SIPC3308	H13-3-DSP2-9- $J_H3^*$	$V_{\kappa}24C-J_{\kappa}4$	$V_{\kappa}1A-J_{\kappa}1$ $V_{\kappa}1C-J_{\kappa}1$ $V_{\kappa}1C-J_{\kappa}2$	
SIPC3301	26.4.1. $\alpha$ -DSP2-9- $J_H3^*$	$V_{\kappa}34C-J_{\kappa}2$	$V_{\kappa}1A-J_{\kappa}1$ $V_{\kappa}1A-J_{\kappa}4$	$V_{\lambda}1-C_{\lambda}1$
SIPC3336	H13-3-DSP2-9- $J_H3^*$	$V_{\kappa}24C-J_{\kappa}4$	$V_{\kappa}1A-J_{\kappa}4$ $V_{\kappa}1C-J_{\kappa}2$ $V_{\kappa}1C-J_{\kappa}4$	$V_{\lambda}1-C_{\lambda}1$
SIPC3282	H13-3-DSP2-9- $J_H3^*$	$V_{\kappa}24C-J_{\kappa}4$	$V_{\kappa}1A-J_{\kappa}2$	$V_{\lambda}1-C_{\lambda}1$

\* $V_{H1}$ -J558 family.

Ig gene assembly is an ordered process that begins with heavy chain gene rearrangement (28) under the regulation of recombinase genes (RAG-1/RAG-2). As the Ig rearrangement process is error prone, only a successful V(D)J assembly leads to pre-B cell receptor assembly (29, 30) and eventual downregulation of RAG-1/RAG-2 activity (31). Further differentiated and proliferating B cells reactivate the recombinases to rearrange the light chain genes, which once again are error prone, i.e., wherein only successful rearrangement leads to maturation rather than cell death (29, 30). Therefore, allelic exclusion, typically the hallmark of Ig gene expression and of an active process, may also stem more from the low frequency of productive or successful rearrangement. With the elucidation of V gene receptor editing (32–38), we now know that lymphocytes expressing self-reactivity in the bone marrow, germinal center (GC), or even in the periphery can be rescued from cell death by exchanging already rearranged V regions with other available V genes through reactivation of the RAG-1/RAG-2 recombination pathway (23, 38–40). It has been shown that recombinase activity is stimulated by low-affinity Abs and is inhibited by high-affinity Abs (41), and accordingly, secondary Ig rearrangements actually occur quite frequently (42–45). RAG-1/RAG-2 activity is also found in GC B cells bearing low-affinity receptors, or in peripheral B-1 cells (23). Indeed, there is increasing evidence that productive and successful light chain rearrangements do not effectively arrest further rearrangement (46–52). Thus, one would then expect frequent violations of allelic exclusion in that  $\kappa:\lambda$  dual expressors (53), as well as dual expressors of  $Ig_{\kappa}$  (54, 55) subtype, should be quite common.

In this study of SIPC tumors, we have observed only a single productive rearrangement of the light chain genes at the Southern hybridization level for  $V_{\kappa}24C-J_{\kappa}4$ . For SIPC3385 (expressing  $V_{\kappa}21G-J_{\kappa}2$ ) and SIPC3301 (expressing  $V_{\kappa}34C-J_{\kappa}2$ ), we find rearrangements in both EcoR1 and BamH1 Southern blots that are, in fact, consistent with predicted sizes from germline restriction maps for both  $V_{\kappa}$  and  $J_{\kappa}$  gene segments (16, 17). We only detected secondarily expressed alleles ( $\kappa$  or  $\lambda$ ) by RT-PCR at levels below the Southern detection levels, i.e., subthreshold. We can eliminate contaminating host tissue as contributing to this observation by several lines of argument. (a) A restricted subset ( $V_{\kappa}1$  or  $V_{\lambda}1$ ) of V genes are found, rather than simply random usage. Furthermore, since  $V_{\lambda}1$  is rarely expressed in the mouse, finding  $V_{\lambda}1$  in 3/5 tumors is highly unlikely. (b) We have recently colinked  $V_{\kappa}24$  and  $V_{\lambda}1$  expression at the single cell level by RT-PCR amplification (our unpublished results). A more likely explanation, therefore, is that we must be observing only a subset of B cells in these tumors expressing both alleles. Thus, the presence of dual expressing  $\kappa:\lambda$  or  $\kappa:\kappa$  alleles indicates that secondary rearrangements are occurring in the SIPC tumors, but whether this rearrangement occurs at significant levels to alter the tumor reactivity patterns must be considered further. Interestingly, it is conceivable that the  $V_{\kappa}24C-J_{\kappa}4$  rearrangement that is shared by three of the tumors may already be an example of secondary rearrangements after a

highly selectable process, as secondary rearrangements of the same allele are often characterized by upstream  $V_{\kappa}$  segments associated with downstream  $J_{\kappa}$  segments. An alternative explanation, that the observed biallelic expression arises from an outgrowth of a subset of tumor cells, cannot formally be discounted. Indeed, when we compare the reactivities (Table II) of two SIPC tumors (SIPC3308, SIPC3336) that exhibit identical  $V_{\kappa}24C-J_{\kappa}4$  and  $V(D)J$  rearrangements, including amino acid substitutions, we find greater reactivity to ssDNA with SIPC3308. Thus, a perceived difference in reactivity between these tumors may stem from the "subthreshold" levels of  $V_{\kappa}1$  or  $V_{\lambda}$ . These "subthreshold" rearrangements do not necessarily have to occur in the bone marrow or GC, but could occur in the periphery where RAG-1/2 can be reactivated (56). However, as recent studies suggest that RAG-1/2 activity may not actually be reinduced, but may reflect differing levels of B cell development (57), we may be observing a small self-renewing B cell population in the tumors presented here. Interestingly, we find RAG-1/2 still active in most of the SIPCs, with the exception of two tumors (SIPC3336 and SIPC3282), both of which also express  $IgV_{\lambda}$ .

The precursor to PCs has long been thought to be the B-1 cell through two lines of evidence: (a) B-1 cells are most abundant in the peritoneum, and are associated primarily with IgA in the lamina propria (58); and (b) in addition to dextran, phosphorylcholine is one of the more common Ags associated with the gut flora and is dominated by the T15 idiotype (1). It has been shown by X-irradiation and failure to regenerate the T15 idiotype by bone marrow reconstitution (59, 60) that the T15 idiotype can only be restored by peritoneal B cells (i.e., B-1 cells). While B-1 cells express Ly1, it is uncertain as to whether

Ly1 is activated as a consequence of immortalization, or whether this represents a distinct B cell lineage. We have determined that several pristane-induced PCs (including M104E and ABPC18) express Ly1 by RT-PCR amplification. Conversely, we have found that the SIPC tumors do not express Ly1, but further studies will be needed to rigorously determine whether the SIPCs are truly B-1a, B-1b, or even B-2 cells.

We now show that the SIPC tumors exhibit secondary Ig light chain gene rearrangements (and accompanying RAG-1/2 activity), exhibit low levels of somatic mutation in  $V_{\kappa}$  or  $V_{\lambda}$ , and show some evidence of clonal heterogeneity. While B-2 cells are mutated and most frequently found in the GC, the B-1 population is most often found in the mantle zone with few somatic mutations (61). The fact that we find evidence of tumors bearing somatic mutations with intracлонаl heterogeneity and with high R/S ratios suggests Ag selection is occurring. V region analysis in several tumor systems besides SIPCs, including Burkitt's lymphoma (62, 63), diffuse large cell lymphoma (64), mantle cell lymphoma (65), and follicular lymphomas (64), demonstrates clonal heterogeneity in that somatic mutations appear to be ongoing during the progression of the tumor. Temporally, many of these tumors arise at different stages of lymphoid maturation and in different lymphoid compartments. In contrast, more mature tumors such as multiple myeloma (66, 67) and pristane-induced mouse PCs (2) have traditionally been found to exhibit homogeneous Abs, suggesting that these transformed cells must have been immortalized post-GC. Based on these findings, we propose that the SIPC tumors may have become an immortalized B cell population in the periphery.

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