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 κ and λ 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)[1] 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, α1,3- and α1,6-dextrans, phosphorylcholine, levan, and β2,1- and β2,6-fructofuranans), a single predominant Ag or hapten target has not surfaced 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

[1] *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 μg 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× SSC at 65°C. The probes used to detect light chain κ rearrangements were either the 1.1-kb HindIII-XbaI (intervening sequence [IVS]) or the 1.8-kb HindIII-XbaI (J_κ) 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, ~1 μg 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_λ, V_k-24, Vκ-1, or C_κ-specific probes. After hybridization, the filters were washed at 0.2× SSC at 65°C for 30 min.

Spectral Karyotyping (SKY™) of SIPC Tumors. Although detailed methods have been reported elsewhere (9), metaphase cells were equilibrated in 2× SSC, digested with RNase A and pepsin, fixed in 1% formaldehyde, denatured in 70% formamide/2× 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× 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® (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 μg 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 λgt10 arms (EcoR1 digested). After ligation, the phage DNA was in vitro packaged and plated to densities of 125,000 per 20 × 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-μl volumes containing 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each dNTP, 2 U of Taq DNA polymerase (GIBCO BRL), 50 pmol of each primer, and a 3 μl 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′), AGGATCTGCATTCTCAGA (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_H-SG1	GTGCAGCTKMAGSAGTCRGG
V_H-SG2	CARCTGCARCARYCTGG
V_H-SG3	GTGAAGCTKSWSGARTCTGG
V_H-SG4	GTYCARCTKCARCAGTCTGG
V_H13-3-5′	GCTGAACTGGCAAAACCT
V_H13-3-3′	GTAATAGACTGCAGAGTC
V_κ1-FW1	AGTCTTGGAGATCAAGCC
V_κ24-CDR1	TCCTGCAGGTCTAGTAAG
V_κ24-FW3	GCCTCAGGAGTCCCAGAC
V_κ1,2	ATGTTGTGATGACCCA
V_κ4,5	AAATTGTTCTCACCCA
V_κ8,14,15,19,21	ACATTGTGMTGACHCA
V_κ9,12,13,31, 33,34	ACATCCAGATGACHCA
V_κ20	AAACAACTGTGACCCA
Vk_κ23	ATATTGTGCTAACTCA
V_κ24,28	RTATTGTGATGACYCA
V_λ	TTCCCAGGCTGTTGTGA
C region primers	
J_H3	GCAGAGAATCTTGGTCCT
IgG3	GTCACTGCAGCCAGGGACCA
IgA	GATGGTGGGATTTCTCGCAGACTC
IgGX	CAGGGGCCAGTGGATAGAC
J_κ2	CCTTAACACTTGATCTGA
J_κ4	CTATTGATGCACAGGTTG
C_κ	ACACTCATTCCTGTTGAA
C_λ	GAGCTCTTCAGAGGAAGG

from the panel. As far as heavy chain class, four out of the five SIPCs 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_H Gene Expression. Initially, Ig heavy chain–specific rearrangements were identified in each tumor by Southern blot analysis using a J_H 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_H 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*

		Antigen activity as measured by ELISA*				
Abs	Isotype	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.

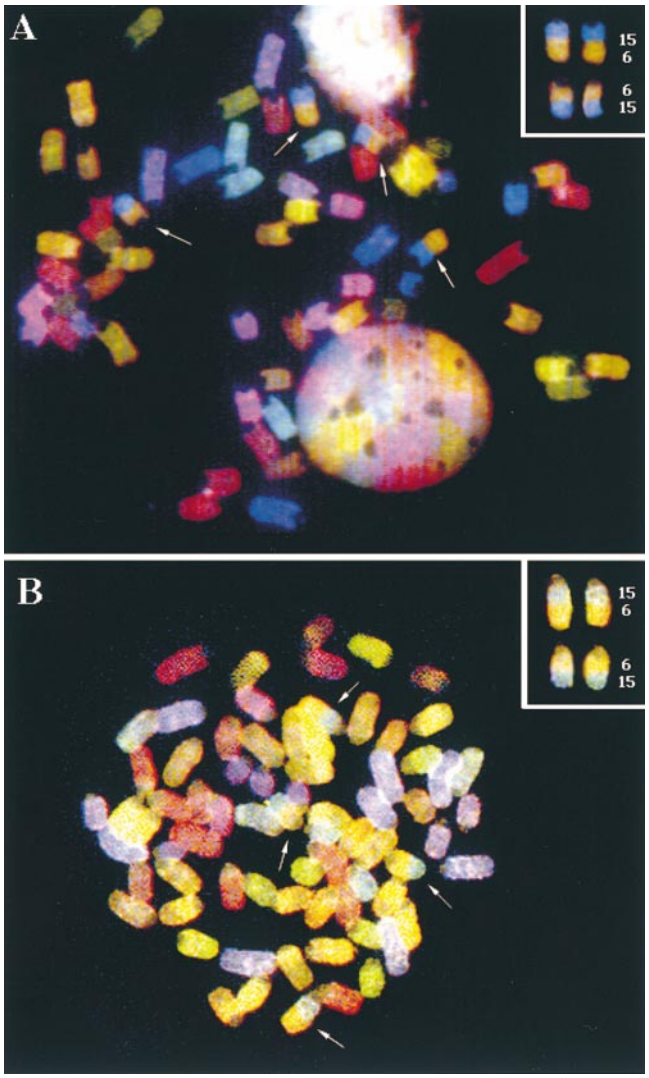


Figure 1. SKY™ images of SIPC tumors with variant T(6;15) chromosomal translocations, (A) SIPC3308 and (B) SIPC3282. The arrows mark chromosomal translocations that have occurred between CHR6 (yellow) and CHR15 (blue). There are two copies of each T(6;15) and the reciprocal T(15;6).

ber of the V_H-J558 family (H13-3; reference 12) rearranged to D_HSP2-9 and J_H3. 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_H3, and use the same D_H region (D_HSP2-9) encoding the amino acid residues Trp-Phe. Although rearranged to J_H3 and D_HSP2-9 as well, SIPC3301 has a longer nucleotide addition sequence, and is in fact encoded by another member (26.4.1-α; 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_H4 and another member of the D_HSP2 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.

Table III. *Cytogenetics of SIPC Tumors*

Tumors	SKY™ analysis	Molecular rearrangement*
SIPC3385	T(12;15)	*c-Myc* (18 kb)
SIPC3308	T(6;15)	NR‡
SIPC3301	T(12;15)	*c-Myc* (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*.

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_κ$24C-$J_κ$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_κ$24C and $J_κ$4, we obtained positive products that were identical as well as nonmutated (Fig. 6). To test whether the $V_κ$24C-$J_κ$4 rearrangement is productive (expressed), we used $V_κ$24C and $C_κ$–specific primers in RT-PCR assays, and again, found positive products in each of

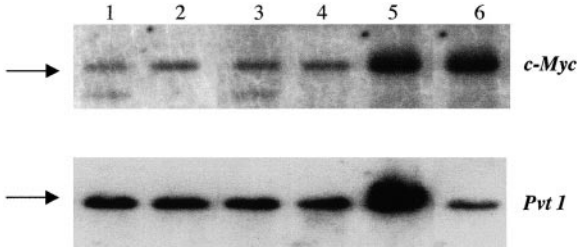


Figure 2. Southern hybridization assay for *c-Myc* and *Pvt 1* rearrangements in SIPC tumors. Shown top (*c-Myc*, EcoR1) and bottom (*Pvt 1*, BamH1) are the following tumors: lane 1, SIPC3385; lane 2, SIPC3308; lane 3, SIPC3301; lane 4, SIPC3336; lane 5, SIPC3282; and lane 6, BALB/c. Arrows depict the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

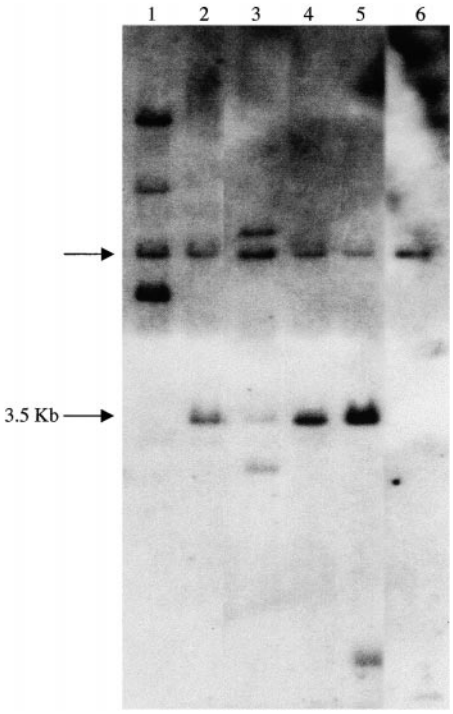


Figure 3. J_H rearrangements in SIPC tumors. Genomic DNAs from SIPC tumors and BALB/c liver were digested with EcoR1 and hybridized with the J_H probe: lane 1, SIPC3385; lane 2, SIPC3308; lane 3, SIPC3301; lane 4, SIPC3336; lane 5, SIPC3282; and lane 6, BALB/c. An arrow (left) depicts the germline (nonrearranged) 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_κ24C-J_κ4, since they are always found in association with the productive rearrangement in each of these tumors. Southern blots with a C_κ 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_κ gene for these two tumors as well. The tumor SIPC3385 was found to express a nonmutated V_κ21G-J_κ2 sequence (16), whereas SIPC3301 expressed a nonmutated V_κ34C-J_κ2 sequence (17).

In the process of verifying the expressed V_κ 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_κ and V_λ gene families. Unexpectedly, we found that in addition to expression of V_κ24C-J_κ4, V_κ21G-J_κ2, and V_κ34C-J_κ2, each of the SIPC tumors expressed an additional Ig_κ or Ig_λ 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_κ1A or V_κ1C (21, 22) rearranged to either J_κ1, J_κ2, or J_κ4 segments

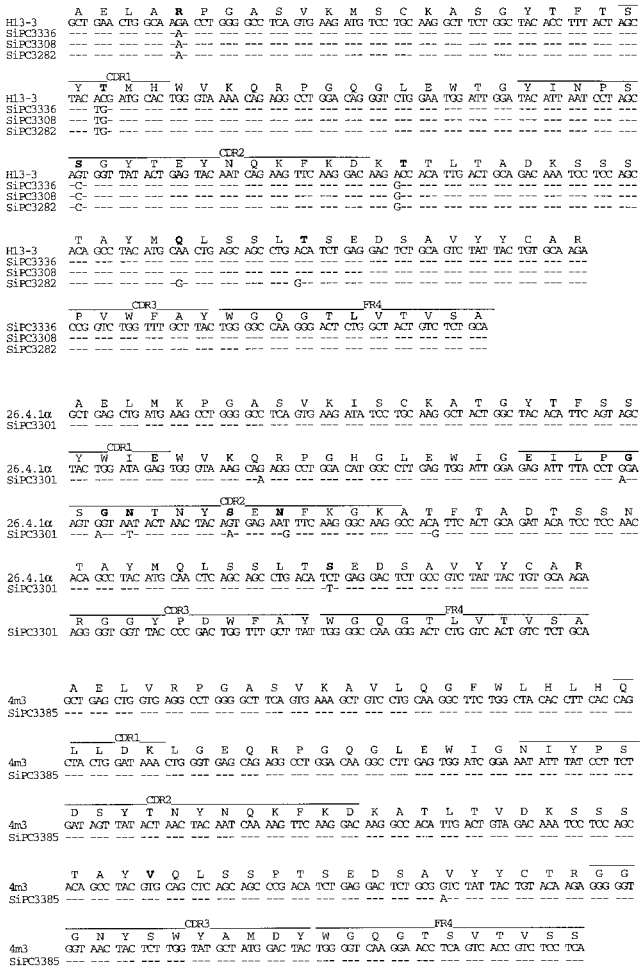


Figure 4. Nucleotide sequences of V_H genes from SIPC tumors. The sequences obtained with the five SIPC tumors have been compared with the most homologous germline genes which are members of the V_H-J558 family. Top: SIPC3336, SIPC3308, and SIPC3282 express V_H13-3; middle: SIPC3301 expresses 26.4.1-α; 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/DDBJ under accession nos. AF154880–AF154882, AF154909, and AF154910.

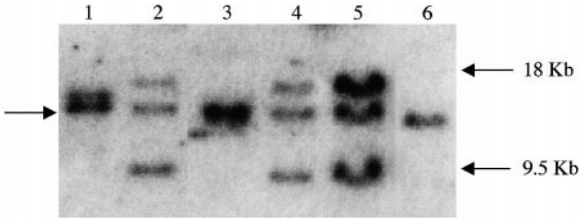


Figure 5. Ig_L rearrangements of SIPC tumors. Genomic DNAs from SIPC tumors and BALB/c liver were digested with EcoR1 and hybridized with the IVS probe: lane 1, SIPC3385; lane 2, SIPC3308; lane 3, SIPC3301; lane 4, SIPC3336; lane 5, SIPC3282; and lane 6, BALB/c. An arrow (left) depicts the germline (nonrearranged)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

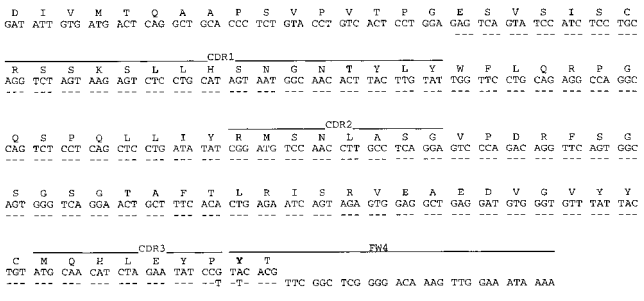
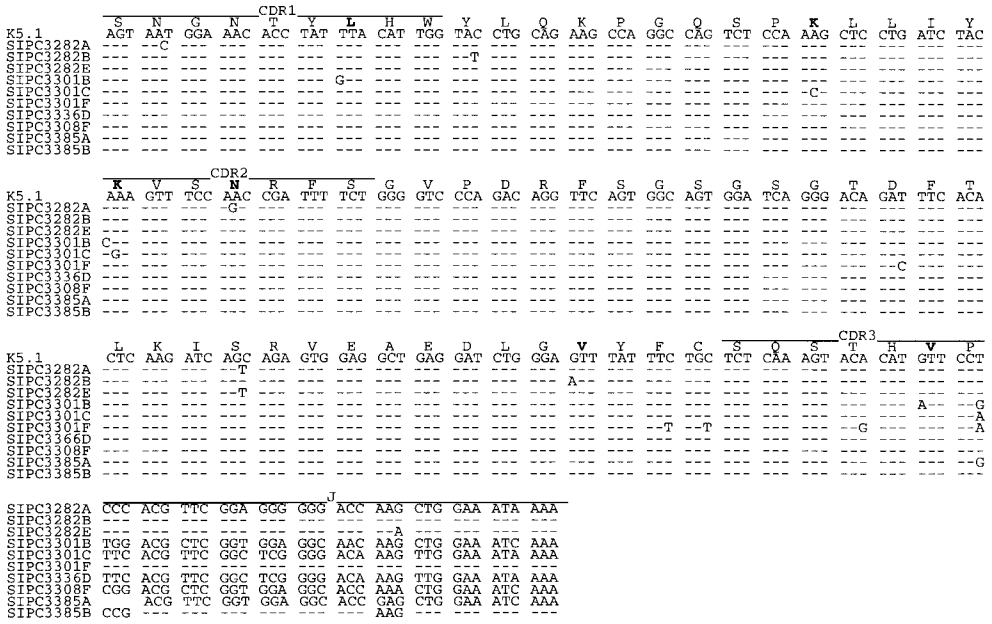


Figure 6. Nucleotide sequence of the V_κ24C-J_κ4 gene from the SIPC tumors. Sequences obtained from SIPC3282, SIPC3336, and SIPC3308 (bottom line) have been compared to that of the germline V_κ24C (reference 15). Dashes indicate sequence identities. Substitutions at the amino acid level are indicated in bold. These sequence data are available from EMBL/GenBank/DDBJ under accession no. AF154911.

(Fig. 7). Furthermore, we also found that both V_κ1C sequences and V_κ1A sequences exhibited some level of somatic mutation (as found in the V_H above). With a 1:1 R/S ratio for the whole V region (and a higher R/S ratio in CDR2) in V_κ1A, only minimal levels of Ag selection are evident. We also found three tumors, SIPC3282, SIPC3301, and SIPC3336, that express V_λ1-C_λ1 sequences (Fig. 8). In this instance, these sequences are mutated (all changes are replacements), and there is evidence of clonal divergence. Interestingly, no V_λ sequences were found in SIPC3385 or SIPC3308. This later result is important, as it is a critical distinction between SIPC3308 and SIPC3336.

Transcript Levels of Ig_κ and Ig_λ in SIPC Tumors. Levels of Ig_κ and Ig_λ were compared by slot blot hybridization using specific probes for V_κ1, V_κ24, V_λ, and C_κ (Fig. 9). Interestingly, SIPC3301 expressed high levels of Ig_λ, whereas SIPC3385 expressed high levels of V_κ1. Consistently, both SIPC3282 and SIPC3336 expressed high levels of both V_κ1



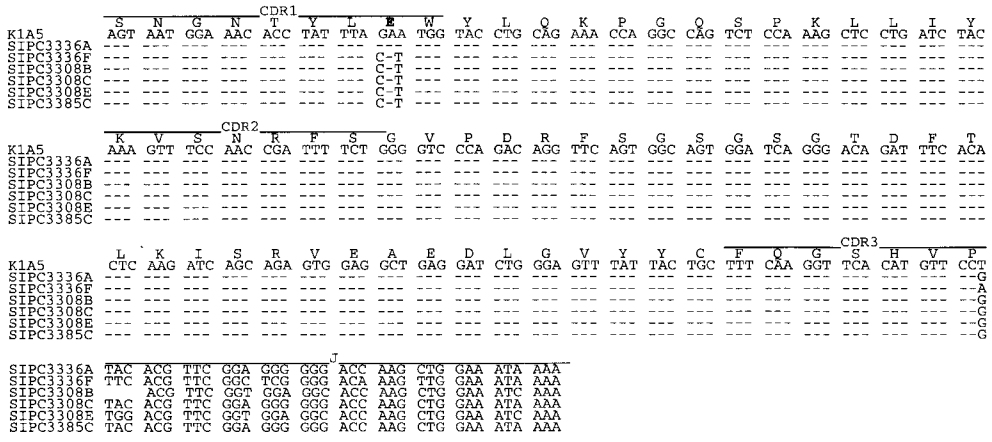


Figure 7. Nucleotide sequences of V_κ1 genes expressed in SIPC tumors. Sequences were obtained from individual clones (designated A–F) of SIPC3282, SIPC3301, SIPC3336, SIPC3308, and SIPC3385. Although multiple subclones with identical sequences were obtained, only representative sequences that differ are shown. The sequences are compared with the most homologous germline gene K5.1 (V_κ1A, top) or K1A5 (V_κ1C, bottom). Dashes indicate sequence identities, and amino acid substitutions are indicated in bold. These sequence data are available from EMBL/GenBank/DDBJ under accession nos. AF154883–AF154898.

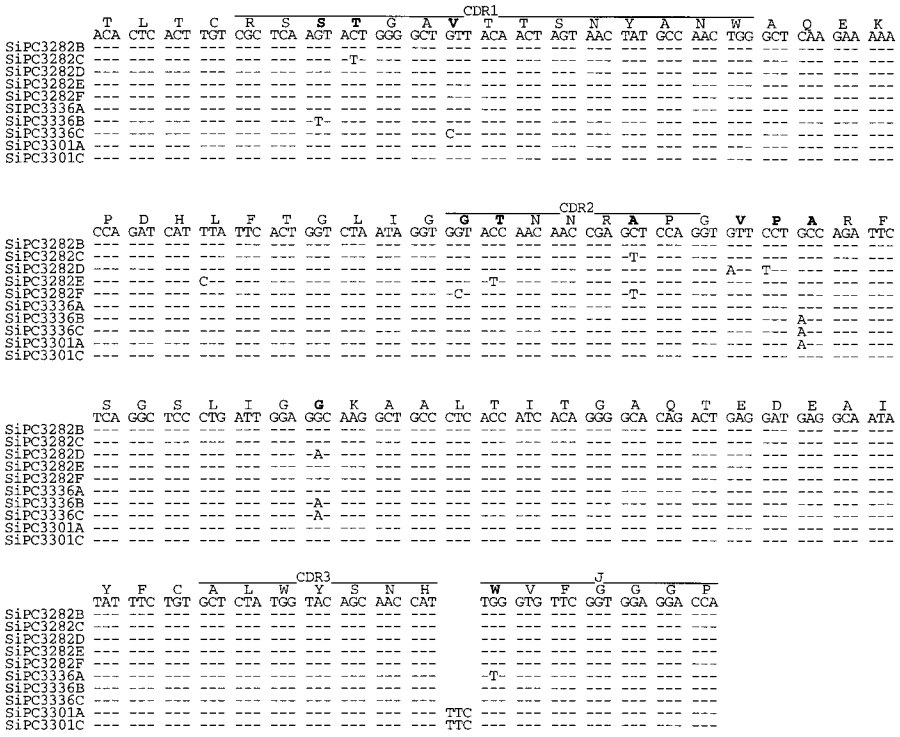


Figure 8. Nucleotide sequences of $V_\lambda 1$ genes expressed in SIPC tumors. Nucleotide sequences of subclones (designated A–F) of V_λ genes from SIPC3282, SIPC3336, and SIPC3301 are compared with the most homologous germline gene. Dashes indicate sequence identities, and amino acid substitutions 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.

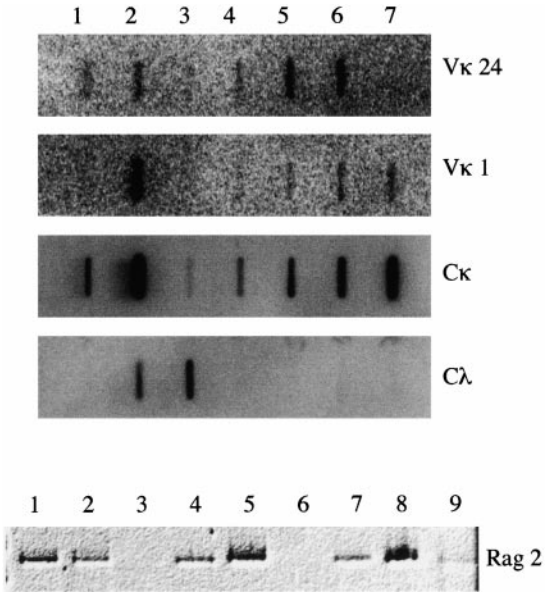


Figure 9. Transcript levels of Ig_κ, Ig_λ, and RAG-2 in SIPC tumors. (Top) Equal amounts of RNA from mouse liver (lane 1), spleen (lane 2), SIPC3301 (lane 3), SIPC3308 (lane 4), SIPC3282 (lane 5), SIPC3336 (lane 6), and SIPC3385 (lane 7) were loaded into slots and hybridized to nick-translated probes from $V_\kappa 24$ (row 1), $V_\kappa 1$ (row 2), C_κ (row 3), or V_λ (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 $V_H 1$ gene member is almost constantly expressed in the case of cryoglobulins expressing the WA recurrent idiotype (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_H-4-39 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_κ21G-J_κ2	V_κ1A-J_κ1 V_κ1C-J_κ2	
SIPC3308	H13-3-DSP2-9-J_H3*	V_κ24C-J_κ4	V_κ1A-J_κ1 V_κ1C-J_κ1 V_κ1C-J_κ2	
SIPC3301	26.4.1.α-DSP2-9-J_H3*	V_κ34C-J_κ2	V_κ1A-J_κ1 V_κ1A-J_κ4	V_λ1-C_λ1
SIPC3336	H13-3-DSP2-9-J_H3*	V_κ24C-J_κ4	V_κ1A-J_κ4 V_κ1C-J_κ2 V_κ1C-J_κ4	V_λ1-C_λ1
SIPC3282	H13-3-DSP2-9-J_H3*	V_κ24C-J_κ4	V_κ1A-J_κ2	V_λ1-C_λ1

*V_H-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 κ:λ dual expressors (53), as well as dual expressors of Ig_κ (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_κ24C-J_κ4. For SIPC3385 (expressing V_κ21G-J_κ2) and SIPC3301 (expressing V_κ34C-J_κ2), 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_κ and J_κ gene segments (16, 17). We only detected secondarily expressed alleles (κ or λ) 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_κ24 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 κ:λ or κ:κ 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_κ24C-J_κ4 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_κ segments associated with downstream J_κ 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_κ24C-J_κ4 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_κ1 or V_λ. 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_λ.

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_κ or V_λ, 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 intraclonal 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.

We wish to acknowledge the continuing support and suggestions of Dr. Michael Potter in the design of these experiments and in critically reviewing the manuscript.

This work was funded in part by a fellowship from the Fogarty Fund.

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Submitted: 2 June 1999 Revised: 9 September 1999 Accepted: 13 September 1999

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