Restricted Immunoglobulin Variable Region (IgV) Gene Expression Accompanies Secondary Rearrangements of Light Chain IgV 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) 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 PVT1 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 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...
have searched for and found polyreactivity to several Ags, including myosin, dsDNA, and ssDNA in BALB/c silic-
cone-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 polyclonal response and possible autoimmunity.

Materials and Methods

Tumors. SIPCs were generated by three successive inocula-
tions (on days 0, 60, and 120) of silicone gel into the peritoneal cavity of BALB/c or BALB/c.DBA/2-Nidh1-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 trans-
planted 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 differ-
ent Ags. After incubation with serial dilutions of each sample in duplicate, peroxidase-conjugated anti–mouse Ig was added; dil-
ung medium alone was included as the negative control. Each assay was done in triplicate.

Southern Hybridization and Slot Blot Analysis.

For slot blot hybridizations, S.91 mg of RNA 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-DBA/2N congenic mouse liver and spleen, as well as from each generation (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).

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Spectral Karyotyping (SKY™) of SIPC Tumors.

Results

SIPC Tumors Display Polyreactivity. The ascites fluid of 33 different SIPCs was assayed 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.
from the panel. As far as heavy chain class, four out of the five SIPC's examined (SIPC 3308, SIPC 3301, SIPC 3336, and SIPC 3282) in this study expressed IgA (only SIPC 3385 expressed IgG3). Although most Abs show higher reactivity to myosin, several are particularly reactive to dsDNA and ssDNA. The tumor SIPC 3301 displays low reactivity with essentially all Ags on this panel. The SIPC 3328, SIPC 3308, SIPC 3336, and SIPC 3385 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 (SIPC 3385, SIPC 3301) 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 SIPC 3385, SIPC 3282, and SIPC 3301. We also found shared rearrangements with both BamH1 (not shown) and EcoR1 digestions in SIPC 3308, SIPC 3336, and SIPC 3282, 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 SIPC 3336, and established that the rearrangement consists of a mem-
The VH-J558 family (H13-3; reference 12) rearranged to DHSP2-9 and JH3. To more specifically determine VH gene usage in the SIPC tumors, we examined the expressed sequences by reverse transcription (RT)-PCR (Fig. 4). Indeed, three of the SIPC 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 aVk24C-Jk4 (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 Vk24C and Jk4, we obtained positive products that were identical as well as nonmutated (Fig. 6). To test whether the Vk24C-Jk4 rearrangement is productive (expressed), we used Vκ24C and Cκ-specific primers in RT-PCR assays, and again, found positive products in each of the three tumors (SIPC3308, SIPC3336, and SIPC3282).

**Ig VL 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 Vκ24C-Jk4 (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 Vκ24C and Jκ4, we obtained positive products that were identical as well as nonmutated (Fig. 6). To test whether the Vκ24C-Jκ4 rearrangement is productive (expressed), we used Vκ24C and Cκ-specific primers in RT-PCR assays, and again, found positive products in each of the three tumors (SIPC3308, SIPC3336, and SIPC3282).

Table III. Cytogenetics of SIPC Tumors

| Tumors   | SKY™ analysis | Molecular rearrangement* |
|----------|---------------|--------------------------|
| SIPC 3385 | T(12;15)      | c-Myc (18 kb)            |
| SIPC 3308 | T(6;15)       | NR ‡                    |
| SIPC 3301 | T(12;15)      | c-Myc (18 kb)            |
| SIPC 3336 | T(6;15)       | NR                      |
| SIPC 3282 | T(6;15)       | NR                      |

*Assayed by digestion with EcoR1 (c-Myc) or BamH1 (Pvt1).
†No rearrangement (NR) with either c-Myc or Pvt1.
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\textsubscript{k}24C-J\textsubscript{k}4, since they are always found in association with the productive rearrangement in each of these tumors. Southern blots with a C\textsubscript{k} 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\textsubscript{k} gene for these two tumors as well. The tumor SIPC3385 was found to express a nonmutated V\textsubscript{k}21G-J\textsubscript{k}2 sequence (16), whereas SIPC3301 expressed a nonmutated V\textsubscript{k}34C-J\textsubscript{k}2 sequence (17).

In the process of verifying the expressed V\textsubscript{k} 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\textsubscript{k} and V\textsubscript{l} gene families. Unexpectedly, we found that in addition to expression of V\textsubscript{k}24C-J\textsubscript{k}4, V\textsubscript{k}21G-J\textsubscript{k}2, and V\textsubscript{k}34C-J\textsubscript{k}2, each of the SIPC tumors expressed an additional Ig\textsubscript{k} or Ig\textsubscript{l} 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\textsubscript{k}1A or V\textsubscript{k}1C (21, 22) rearranged to either J\textsubscript{k}1, J\textsubscript{k}2, or J\textsubscript{k}4 segments.
Ig Genes and Secondary Rearrangements in Plasmacytomas

Furthermore, we also found that both $V_{\kappa}$1C sequences and $V_{\kappa}$1A sequences exhibited some level of somatic mutation (as found in the $V_{\lambda}$ above). With a 1:1 R/S ratio for the whole $V$ region (and a higher R/S ratio in CDR2) in $V_{\kappa}$1A, only minimal levels of Ag selection are evident. We also found three tumors, SIPC 3282, SIPC 3301, and SIPC 3336, that express $V_{\lambda}$1-C,1 sequences (Fig. 8). In this instance, these sequences are mutated (all changes are replacements), and there is evidence of clonal divergence. Interestingly, no $V_{\kappa}$ sequences were found in SIPC 3385 or SIPC 3308. This later result is important, as it is a critical distinction between SIPC 3308 and SIPC 3336.

Transcript Levels of Ig$\kappa$ and Ig$\lambda$ in SIPC Tumors. Levels of Ig$\kappa$ and Ig$\lambda$ were compared by slot blot hybridization using specific probes for $V_{\kappa}$1, $V_{\kappa}$24, $V_{\lambda}$, and C$\kappa$ (Fig. 9). Interestingly, SIPC 3301 expressed high levels of Ig$\kappa$, whereas SIPC 3385 expressed high levels of $V_{\lambda}$. Consistently, both SIPC 3282 and SIPC 3336 expressed high levels of both $V_{\kappa}$1

Figure 6. Nucleotide sequence of the $V_{\kappa}$24C-J4 gene from the SIPC tumors. Sequences obtained from SIPC 3282, SIPC 3336, and SIPC 3308 (bottom line) have been compared to that of the germline $V_{\kappa}$24C (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.

Figure 7. Nucleotide sequences of $V_{\lambda}$ genes expressed in SIPC tumors. Sequences were obtained from individual clones (designated A-F) of SIPC 3282, SIPC 3301, SIPC 3336, SIPC 3308, and SIPC 3385. 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_{\lambda}$1A, top) or K1A5 ($V_{\lambda}$1C, 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.
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.
We have found that three independent SIPC tumors, each derived from different generations (SIPC 3282, SIPC 3308, and SIPC 3336), 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) SIPC 3282 shows an additional rearrangement (J_{H}2) not found in other tumors, as well as additional somatic mutations not found in other tumors; (d) SIPC 3385 and SIPC 3308 both lack V_{L}1 rearrangements; and (e) no RAG-1/2 expression is found in SIPC 3282 or SIPC 3336. 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_{L}1 gene member is almost constantly expressed in the case of cryoglobulins expressing the WA recurrent idioype (60% of cases), and the V4-34 (V_{L}4-21) gene is constantly expressed by cold agglutinins with anti-I specificity (95% of cases [25]). Although the DH region is not identical among these Abs, a report by Fais et al. (26) shows that several Abs and J_{H}3-3 region is used. In addition, a report from the same group indicated that five different cases of CD5^{+}Ig^{+} CLLs expressed virtually identical Ag receptors, by recombining (unmutated) the V_{H}4-39 gene to D6-13-J_{H}5b and the V_{L}012 gene to J_{L}1 (27).

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

| Tumors | Expressed heavy chain | Expressed light chain |
|--------|-----------------------|-----------------------|
|        |                       | 1  | 2  | 3  |
| SIPC 3385  | 4m3-                  | V_{L}21G-J_{L}2       | V_{L}1A-J_{L}1       | V_{L}1C-J_{L}2 |
|          | DSP2-                 |    |    |    |
|          | J_{H}4*               |    |    |    |
| SIPC 3308  | H13-3-                | V_{L}24C-J_{L}4       | V_{L}1A-J_{L}1       | V_{L}1C-J_{L}2 |
|          | DSP2-9-               |    |    |    |
|          | J_{H}3*               |    |    |    |
| SIPC 3301  | 26.1 alpha            | V_{L}34C-J_{L}2       | V_{L}1A-J_{L}1       | V_{L}1C-J_{L}1 |
|          | DSP2-9-               |    |    |    |
|          | J_{H}3*               |    |    |    |
| SIPC 3336  | H13-3-                | V_{L}24C-J_{L}4       | V_{L}1A-J_{L}4       | V_{L}1C-J_{L}1 |
|          | DSP2-9-               |    |    |    |
|          | J_{H}3*               |    |    |    |
| SIPC 3282  | H13-3-                | V_{L}24C-J_{L}4       | V_{L}1A-J_{L}2       | V_{L}1C-J_{L}1 |
|          | DSP2-9-               |    |    |    |
|          | J_{H}3*               |    |    |    |

*V_{H}1-J558 family.
highly selectable process, as secondary rearrangements of the same allele are often characterized by upstream V\_k segments associated with downstream l\_k 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 activities (Table II) of two SIPC tumors (SIPC3308, SIPC3336) that exhibit identical V\_k,24C\_l,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\_1 or V\_\_k. 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 SIPC\_k, with the exception of two tumors (SIPC3336 and SIPC3282), both of which also express IgV\_k.

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 primarily with IgA in the lamina propria (58); and (b) in addition to dextran, phosphorylcholine is one of the more common Ags associated with gut flora and is dominated by the T15 idiotype (I). 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 R T-PCR amplification. Conversely, we have found that the SIPC tumors, including Burkitt’s lymphoma (62, 63), diffuse large cell lymphoma (64), mantle cell lymphoma (65), and follicular lymphomas (64), demonstrate 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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