

# Evidence for an Antigen-driven Humoral Immune Response in Medullary Ductal Breast Cancer<sup>1</sup>

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## ABSTRACT

A minority of breast cancers is characterized by lymphoplasmacytic infiltrates that have been correlated with improved patient survivals. The association of improved prognosis with plasmacytic infiltrates has been classically linked with the rare medullary carcinoma subtype but is also evident in the smaller infiltrated fraction of the more abundant nonmedullary (not otherwise specified) tumors. It is our hypothesis that these plasma cell (PC) infiltrates represent a host humoral response driven by one or more tumor-derived neoantigens. As the index study group, two primary medullary carcinoma tumors were examined. Immunophenotyping confirmed a large number of IgG PCs in contradistinction to normal breast, which typically contains a lesser number of mainly IgA isotypes. IgG heavy and light chains were expressed as combinatorial phage Fab libraries. VH and VL sequences showed a preponderance of clonal groups in both patients, as identified by germ-line gene usage and junctional mutation patterns. Panning of phage Fab libraries against purified antigens excluded Her2/*neu* and p53 as the eliciting antigen, and failure of clonal enrichment by cell panning suggested that the neoantigen was not membrane expressed or was expressed at low levels. Cognate, original VH+VL pairs were obtained by single cell PCR of tumor PCs, which showed overlap with the pooled IgG libraries. Tumor-derived IgG V genes exhibited mutational patterns that were consistent with antigenic selection and affinity maturation. Where examined, IgG1 was the predominant isotype, consistent with a T-dependent (*i.e.*, protein) antigen. From these data, we infer that the breast tumor PC infiltrates of the medullary carcinoma subtype are compatible with an autogenic tumor neoantigen-driven humoral immune response.

## INTRODUCTION

In the search for new approaches in the understanding and treatment of malignancies, it has been proposed by Lippman (1) that “the most appropriate protein targets may emerge from a consideration of prognostic variables . . . shown to be of value in clinical practice.” The present study was undertaken to build on this premise, to explore the molecular basis for the apparently improved survival in PC<sup>3</sup>-infiltrated breast carcinomas. Breast cancer is remarkable among human malignancies in the incidence of lymphoplasmacytic infiltrates in a minority of tumors that suggest an ongoing humoral immune response. This finding has been considered characteristic of the med-

ullary ductal carcinoma (MC) subtype, but PC-infiltrates are also present in a fraction of nonmedullary (NOS) infiltrating ductal carcinomas that may reflect similar etiologies.

MCs are relatively rare breast tumors, diagnosed in up to 5% of cases (2). Grossly, they are circumscribed without encapsulation and are infrequently bilateral. MC is also circumscribed microscopically, but its appearance is otherwise highly ominous, with large cells, abundant cytoplasm, large bizarre nuclei, and frequent mitoses. Virtually all are histologic nuclear grade III, usually the worst prognostically, and they display a high degree of aneuploidy and typically lack hormone receptors. Yet patients with MC often do better than predicted for size, grade, and lymph node status after surgery-only therapy (3–8). [For understanding the natural history of PC-infiltrated breast carcinomas, we prefer the mainly older literature on “surgery-only” treatment, because chemotherapies may have more impact on an immune response than on a resistant tumor, complicating for our purpose the otherwise excellent studies that include chemotherapies (*e.g.*, Ref. 9).] The tumor is infiltrated and surrounded with lymphocytes and plasma cells; in its most exuberant expression, it was classically designated “medullary carcinoma with lymphoid stroma.” Similarly, the presence of a PC infiltrate in NOS tumors is also favorable prognostically, suggesting that the PC reaction is the basis for the improved survival rather than the tumor histologic type. Ridolfi *et al.* (4) specifically examined the role of PCs in 192 cases of patients with MC in comparison with those with NOS and showed a correlation of increased PC infiltration with better survival in MC and NOS tumors alike, prompting the oft-stated impression that these tumors may be regulated by a host immune response. Finally, MC patients who failed primary therapy died the fastest of all of the groups (5), seemingly reflecting the aggressive histology of MC and the high proliferative index when the immune reaction was ineffective, again reinforcing the role of the immune response rather than the histology as determining the favorable prognosis.

As to the origin of the immune response, the infiltrates have been noted to be confined to the tumor bed itself without extending into the adjacent normal breast tissue, suggesting “some maladjustment between tumor and host” (3). The PCs in MC and infiltrated NOS were disproportionately of the IgG type, whereas uninvolved or contralateral breast tissue contained far fewer PCs, which were of the IgA type, as is typically associated with normal secretory epithelium (Refs. 10–12; a fourth reported IgG bias for a subset of MC; Ref. 13), leading to the speculation, “the stronger the stimulation by tumor-associated antigens, the higher the proportion of IgG” plasma cells (9). The antigenic phenotypes of the inflammatory infiltrates have been called “essentially similar” between MC and NOS (11, 14, 15), emphasizing again the centrality of the immune reaction rather than the tumor histologic type. The prominence of IgG *versus* the usual IgA plasma cells in MC and infiltrated NOS tumors alike, and the confinement of reactions to the tumor beds, suggest a specific response to one or more tumor-derived neoantigens in the tissues.

It is the primary hypothesis of this study that the diagnostic lymphoplasmacytic infiltrate typically observed in MC and less frequently in NOS tumors represents a matured, tumor-specific autogenic humoral immune response. The distinction between NOS-infiltrating

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<sup>3</sup> The abbreviations used are: PC, plasma cell; MC, medullary carcinoma; NOS, not otherwise specified; TMC, typical medullary carcinoma; Ig, immunoglobulin; Hc, heavy chain; Lc, light chain; SC, single cell; MAT, murine anti-Tac; anti-Id, anti-idiotype; RT-PCR, reverse transcription-PCR.

ductal and MC histologically may be somewhat artificial in that the tumors likely represent a continuum, as evidenced by the interobserver variability and controversies in pathologic diagnosis (16–18), with the more important feature for our purpose being the presence of the PC infiltrates that is more common in MC as part of its diagnostic criteria. With this caveat in mind, as our index effort to address this question we selected two tumors of the MC subtype for initial examination by histological and molecular criteria for indicators of an antigen-driven humoral response.

Hallmarks of B-cell activation, differentiation, and proliferation were tracked by analysis of IgG sequences from both pooled sequences in combinatorial libraries and from single tumor-infiltrating PCs. Our data demonstrate clonal groups of immunoglobulin genes and confirm features consistent with the hypothesized antigen-driven tumor-derived B-cell immune response. These data suggesting an eliciting tumor neoantigen, with the clinical observation of improved prognosis in PC-infiltrated tumors, may, in turn, suggest that the antibodies secreted by these PCs may have an autoimmunotherapeutic antitumor benefit in these patients.

## MATERIALS AND METHODS

### Proteins

Purified Her2/*neu* was obtained from Dr. B. Fendly (Genentech, South San Francisco, CA). p53 was obtained as a glutathione *S*-transferase-fusion protein from Dr. Dutta (Children's Hospital, Boston, MA). Murine anti-Tac antibody was obtained from Dr. T. Waldmann (National Cancer Institute/NIH, Bethesda, MD).

### Tumors and Cells

MC tumors were obtained as surgical discard samples under approval of the Institutional Review Board of the Beth Israel Deaconess Medical Center. A fresh, unfrozen tumor was manually disaggregated and purified by Ficoll gradient centrifugation (Histopaque 1077; Sigma). Few viable tumor cells survived the procedure; the purification yielded mainly mononuclear cells (plasma cells and lymphocytes), which retained good viability. Cells ( $5 \times 10^7$ ) were placed in solution D [6 M guanidinium isothiocyanate (Life Technologies, Inc.) containing 0.1 M  $\beta$ -mercaptoethanol] for combinatorial library preparation (below), and the remainder were stored viably in aliquots of  $5\text{--}10 \times 10^6$  cells in liquid nitrogen.

Tissues samples were processed for histology and immunohistochemistry (IgA, IgG, IgM, Ig- $\kappa$ , and Ig- $\lambda$ ) in the Pathology Department of the Beth Israel Deaconess Medical Center. Diagnosis of medullary carcinoma and classification into subgroups was based on standard criteria, including syncytial growth pattern, circumscribed margins, mononuclear infiltrate, grade 2 or 3 nuclei, no intraductal component, and no microglandular differentiation (4). Presence of all of these features is indicative of TMC, whereas tumors with two or more atypical features are classified as atypical MC or non-MC. The survival advantage is most evident in the TMC group (4, 18).

Patient 1 (GK): Primary tumor cells were poorly differentiated, and the tumor had a heavy lymphoplasmacytic infiltrate. There was no duct formation, and growth pattern was syncytial. In most areas the tumor was circumscribed, but it extended focally into the surrounding breast tissue. On the basis of histological features, the diagnosis for this patient was TMC. Tumor was estrogen receptor negative.

Patient 2 (PT): Primary tumor cells were poorly differentiated, and the tumor was circumscribed with heavy lymphoplasmacytic infiltrate. The tumor was diagnosed as TMC based on histological features.

MC cell line HTB-24 (American Type Culture Collection, Bethesda, MD) and human renal cell carcinoma line 293 (American Type Culture Collection) were maintained in RPMI 1640 supplemented with 10% FCS.

### Library Construction

**Combinatorial.** Phage Fab display libraries were constructed from MCs from two patients designated patient 1 (GK library) and patient 2 (PT library)

as described by Burton and Barbas (19). RNA was isolated from  $5 \times 10^7$  lymphoplasma cells stored frozen in solution D (above) by phenol-chloroform extraction and isopropanol precipitation. RNA was reverse transcribed using Superscript reverse transcriptase (Life Technologies, Inc.), and oligodeoxythymidylate (Perkin Elmer). Ig heavy and light chains were amplified by PCR as described (20). Three reactions were run for each sample, with one each for  $\lambda$  Lc,  $\kappa$  Lc, and  $\gamma$ 1 Hc. Lc primers were used as described, with sets of 5' Ig V consensus primers specific to  $\kappa$  Lc V gene families or  $\lambda$  Lc V gene families. Patient 1 (GK) Hcs were amplified using a consensus 5' primer (VHGK) with the sequence: AGGTGCAG(CTCGAG)CAGTCTGG and a VH4 primer with the sequence: CCG(CTCGAG)CAGGTGCAGCTGCAGGAGTCSG with the restriction cloning site marked. Patient 2 (PT) heavy chains were amplified with a mixture of 5' Ig V consensus primers specific to Hc V gene families as described in Burton and Barbas (15). Constant region primers (3') were specific to IgG1 Hc (CG1Z), and  $\lambda$  (CL2) or  $\kappa$  (CK1Z) Lcs.

PCR products were prepared and cloned into a Fab phage display vector as described in Kingsbury *et al.* (20) using the NPC3 M13 surface display system (gift of Dr. Carlos Barbas, Scripps Research Institute, La Jolla, CA) and VCSM13 helper phage (Stratagene). Phagemid isolates containing randomly paired Hcs and Lcs (1 Hc and 1 Lc per isolate) were selected for double inserts as described previously (21). Fifteen clones containing  $\lambda$  Lc and  $\gamma$ 1 Hc, and 15 clones containing  $\kappa$  Lc and  $\gamma$ 1 Hc were selected randomly and sequenced from each patient for a total of 30 phagemid clones/patient.

**Single Cell.** An SC library was prepared from the PCs of patient 1. Stored lymphoplasma cells were thawed and stained with FITC-conjugated mouse antihuman CD38 (Caltag). CD38<sup>hi</sup> PCs (22) were sorted as SC into wells of 96-well PCR plate and amplified by RT-PCR with two rounds of nesting (PCR1 and PCR2), as described previously (23). Three sets of PCR1 reactions were run per cell: one for  $\lambda$  Lc, one for  $\kappa$  Lc, and one for  $\gamma$  ( $\gamma$ 1) Hc. PCR1 employed pooled 5' V family primers and a constant domain primer. PCR2 employed a nested constant primer and single V family primers. Single primer products from PCR2 were purified (Qiaquick; Qiagen) and directly sequenced. Multiple PCR products were sequenced from most single PCs.

### DNA Sequencing

Sequencing was done both by dideoxy termination using Sequenase 2.0 from USB and by automated sequencing of PCR-amplified Hc and Lc inserts (ABI Prism dye termination system; Perkin Elmer). IgV genes was amplified for direct sequencing using PCR Ready-To-Go beads (Pharmacia) in a Perkin Elmer Thermocycler in a cycle of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. NPC3-specific primers for the amplification of inserts from the combinatorial library were used for sequencing (20).

Germ-line gene usage was determined by comparison to the IMGT database of Ig sequences<sup>4</sup> using the DNAPLOT alignment tool (24) and by visual examination of sequences. Numbering and gene designations are as described for IMGT. Clonality was assessed on the basis of shared germ-line gene usage between sequences, mutated characters, and junctional mutation pattern, as determined by CLUSTALV alignment and examination of V(D)J junctions. Comparisons with germ-line genes for percentage of mutation were done for FR1, CDR1, FR2, CDR2, and FR3 regions only, and excluded the CDR3 portion of the V gene because of the high incidence of deletions in this region. Deletions were not scored as mutations for the purposes of total percentage of identity.

### PCR Error Rate

IgG1 CH1 sequence from randomly selected SC and combinatorial library clones were compared to the germ-line IgG CH1 sequence to determine the PCR error rate for combinatorial (0.4% after 35 cycles) and SC (0.18% after 70 cycles) library sequences. This would result in 1.1 mutations on average per Ig V gene in the combinatorial library but with no effect on the SC final sequences, which were resolved from multiple sequences and, thus, were error free (25, 26). All of the PCR errors were A > G or G > A transitions, as noted previously (27).

<sup>4</sup> Internet address: <http://imgt.cnusc.fr:8104>.

## Panning

**Purified Antigen-based Panning.** EIA/RIA plates (Costar) were coated with 50  $\mu$ l-well of a solution of 5  $\mu$ g/ml antigen in 0.1 M NaHCO<sub>3</sub> (pH 8.5) overnight at 4°C, and blocked as described previously (20). Antigens were Her2/*neu*, p53, and MAT antibody ("Proteins," above). Phage Fab suspensions containing 10<sup>12</sup> pfu in 25  $\mu$ l were added to each well in quadruplicate and incubated for 2 hrs at 37°C. Phage were libraries or single clones of MC tumor-derived phage Fab, human anti-Id phage Fab clone (reactive with MAT) as positive control (20), or human anti-tetanus toxoid phage Fab as negative control. After incubation, phage were aspirated, the wells were washed, and bound phage was eluted, titered, and replicated as described previously (28).

**Cell-based Panning.** HTB24 medullary breast carcinoma cells and 293 embryonal kidney cells (negative control) were used in cell-based panning experiments as live or fixed cells as described earlier (29). Fixed cells were incubated with phage in PBS/10% horse serum. Live cells were incubated with phage in the appropriate growth medium, *i.e.* RPMI/10% FCS. Phage were eluted from the cells and replicated as described previously (28, 29). Eluted phage were compared with input phage by Alu "fingerprint" analysis for evidence of repertoire shift after cell panning (29).

**Flow Cytometry.** HTB24 and 293 cells were incubated with phage Fab with 10<sup>12</sup> phage in 0.1 ml of binding buffer (RPMI 1640 + 10% horse serum) for 30 min on ice, washed, and stained with anti-M13-FITC, then examined by cytofluorometry on a Coulter Epics II flow cytometer.

## RESULTS

The aim of the present study was to evaluate whether the infiltration of lymphocytes and plasma cells, characteristic of MC, is attributable to an antigen-driven tumor-specific immune response. Two primary medullary breast tumor specimens were examined. Both samples had classic features with abundant infiltration of lymphocytes and PCs. Tissues were immunophenotyped and prepared as SC suspensions and immediately processed for M13 phagemid cloning or stored for later SC analysis. Hallmarks of an antigen-driven, tumor-specific immune response would be: (a) IgG *versus* IgA dominated; (b) a focused immune repertoire; (c) abundant somatic mutations concentrated in the antibody CDRs; and, ultimately, (d) antigen reactivity. These features were examined.

### IgG Is the Dominant Isotype of Infiltrating PC Ig

Paraffin-embedded primary medullary carcinoma from patient 1 was sectioned, H & E stained, and immunohistochemically stained for IgA, IgG, and IgM (Fig. 1). Infiltrating cells were predominantly IgG with some IgA and few IgM. In separate, approximately matching fields, positively-staining cells were tallied as 207 IgG, 56 IgA, and 27

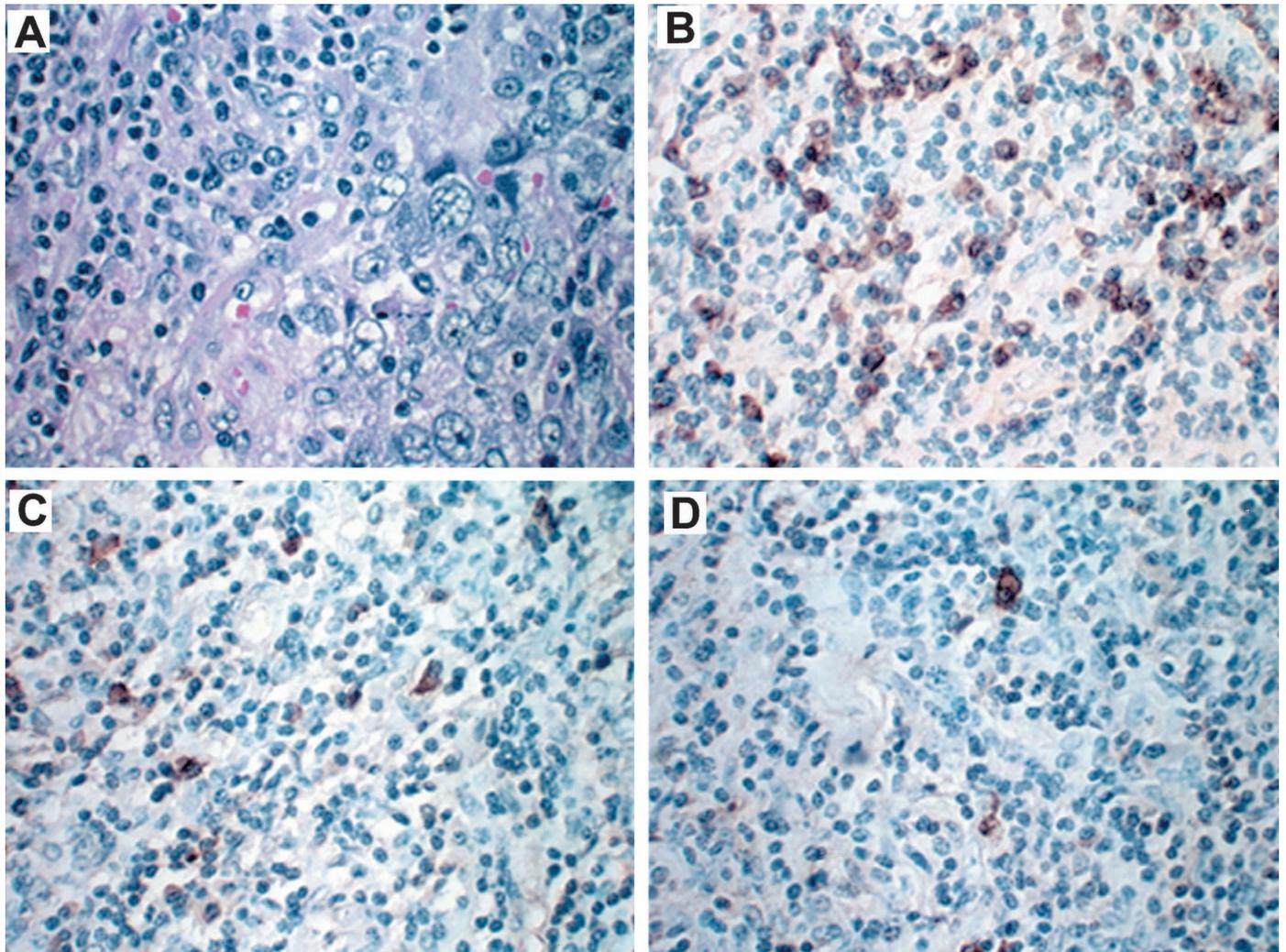


Fig. 1. Immunophenotype of MC tumor-infiltrating plasma cells. A, primary tumor from patient 1 was paraffin-embedded and stained with H & E. Plasma cells and lymphocytes are visible as small round cells with darkly staining nuclei, more prominent on the left of the photograph. MC cells are large irregular cells with large, variegated-staining nuclei. Sections were stained immunohistochemically for (B) IgG, (C) IgA, and (D) IgM. Brown staining indicates Ig positivity. High levels of cytoplasmic Ig in PCs makes this technique more sensitive for PCs than B cells.

Table 1 *Germ-line Hc V gene usage and clonal groups of patient 1*

Hc sequences for the GK combinatorial libraries were analyzed by comparison to germ-line Ig VDJ genes using the IMGT database. Sequence designations follow the IMGT numbering system, in which germ-line genes are grouped by family number, followed by a dash then germ-line gene, both numbered according to chromosomal location. Allele is indicated by \* and number. Clone numbers designate phagemid clone containing Lc (GKK/GKL#). Sequences are grouped by common V gene usage. Clonal groups, as identified by common VDJ germ-line gene usage and junctional mutation, are boxed.

Clonal group	Clone	V gene	D gene	J gene
1	GKL3 HC	IGHV5-51*01	IGHD4-23*01	IGHJ3*02
	GKL20 HC	IGHV5-51*01	IGHD4-23*01	IGHJ3*02
	GKK14 HC	IGHV5-51*01	IGHD4-23*01	IGHJ3*02
	GKK139 HC	IGHV5-51*01	IGHD4-23*01	IGHJ3*02
	GKL55 HC	IGHV5-51*01	IGHD4-23*01	IGHJ3*02
	GKK15 HC	IGHV5-51*01	IGHD4-23*01	IGHJ3*02
	GKK69 HC	IGHV5-51*01	IGHD5-18*01	IGHJ3*02
	GKL41 HC	IGHV5-51*01	IGHD3-3*01	IGHJ4*02
	GKK137 HC	IGHV5-51*01	IGHD5-18*01	IGHJ4*02
	GKK61 HC	IGHV5-51*01	IGHD6-19*01	IGHJ4*02
2	GKL27 HC	IGHV5-51*03	IGHD2-15*01	IGHJ4*02
	GKK129 HC	IGHV5-51*03	IGHD2-15*01	IGHJ4*02
	GKK22 HC	IGHV5-51*03	IGHD3-3*02	IGHJ4*02
3	GKL26 HC	IGHV5-51*03	IGHD3-10*01	IGHJ6*02
	GKL61 HC <sup>a</sup>	IGHV1-18*01	IGHD6-19*01	IGHJ4*02
4	GKK16 HC	IGHV1-18*01	IGHD6-19*01	IGHJ4*02
	GKL34 HC	IGHV1-18*01	IGHD1-26*01	IGHJ4*02
5	GKK11 HC	IGHV5-a*01	IGHD5-24*01	IGHJ6*02
	GKK118 HC	IGHV5-a*01	IGHD5-24*01	IGHJ6*02
	GKL18 HC	IGHV5-a*01	IGHD5-24*01	IGHJ6*02
6	GKL35 HC	IGHV5-a*01	IGHD6-25*01	IGHJ4*02
	GKL38 HC	IGHV5-a*01	IGHD6-25*01	IGHJ4*02
6	GKK88 HC	IGHV1-2*02	IGHD3-16*01	IGHJ4*02
	GKL4 HC	IGHV1-2*02	IGHD3-16*01	IGHJ4*02
	GKL11 HC <sup>a</sup>	IGHV4-61*02	IGHD6-25*01	IGHJ6*02
	GKL64 HC	IGHV4-30-1/4-31*02	IGHD5-12*01	IGHJ4*02
	GKK86 HC	IGHV1-e*01	IGHD2*02	IGHJ3*02
	GKL10 HC	IGHV3-11*01	IGHD1-26*01	IGHJ6*02

<sup>a</sup> Members of clonal groups with SC library members.

IgM. Furthermore, whereas the IgA and IgM staining cells were predominantly mature plasma cells, the IgG+ cells included lymphoblasts, lymphoplasmablasts, and plasma cells, representing all stages of differentiation of the IgG B cell lineage, compatible with ongoing recruitment and B-cell differentiation in response to a locally produced antigen. A similar phenotype was evident for the tumor of patient 2. These results are consistent with observations published previously of PC-infiltrated MC and NOS breast tumors (10–13).

**Antibody Repertoire Focus**

The sources of Ig mRNA for this analysis were mononuclear cell fractions from disaggregated primary tumors that contained a mixture of lymphocytes and PCs. Because of the vastly larger amount of Ig mRNA per cell in PCs versus B cells (up to ~1000-fold greater), this mRNA-based cloning method effectively samples the most matured, PC-component of these infiltrates and is referred to as such in this presentation.

Libraries were initially prepared in a vector in which the VH and VL are displayed as Fab molecules on the tail proteins of M13 phage (phage Fab). This cloning method provided a sample of IgG VH heavy chains that are randomly paired with Vκ or Vλ light chains, as a so-called combinatorial library (30); both Lc libraries are paired independently against the same IgG Hc library. No single IgG primer captures the four subtypes and preserves the interchain disulfide configurations as needed for proper Fab assembly for phage display. Instead, IgG1 has come to be the default choice, because it typically dominates other subtypes *in vivo* and is the subtype of IgG that is most associated with T-dependent (*i.e.*, protein) antigens (31).

The phage display format permitted analysis of the antibody repertoire focus by direct sequencing but also had potential advantages in

antigen identification. Phage Fab enables screening for reactivity by “panning” against antigens as purified proteins (19, 20) and on cells (29, 32–34), and also permits antigen isolation by immunoprecipitation (28). Because this format randomizes the association of VH and VL, reactivity serves to define VH-VL pairs that are “antigen-competent” if not actually recreating the original plasma cell/B-cell pairing (35).

If the PC infiltrate is the result of an eliciting antigen within the tumor, this would be reflected by a restricted (focused) repertoire. Clonality was evaluated by sequencing of the library immunoglobulin V genes. Phagemid clones (~15) with λ Lcs, and ~15 phagemid clones with κ Lcs were randomly selected and sequenced from the separate combinatorial libraries for a total of 30 Hcs and 30 Lcs from each patient (Tables 1–4).

Definitive identification of clonal relatives was by comparison of V(D)J rearrangements and junctional mutation patterns. In general, unique specification of VDJ for Hc was enough to indicate clonality in this sample, whereas specification of VJ alone for Lc was frequently insufficient to specify clonality. The lower combinatorial possibilities and biases in J chain selection make the VL combinations more likely to be repeated on a random basis than the VHs. In this case, when VJ genes were the same, distinct clonal origins were nonetheless evident by the junctional mutation differences (Fig. 2). On the other hand, the possibility of phage clone duplications (“sibling clones”) was ruled out in these samples by the presence of different VH or VL partners in each different phage Fab (not shown) and by the different somatic mutations (below) that were present in the different isolates. Therefore, clonality in these samples is an authentic reflection of the tumor specimen and not an artifact of the cloning method.

Table 2 *Germ-line Lc V gene usage and clonal groups of patient 1*

Lc sequences for the GK combinatorial libraries were analyzed by comparison to germ-line Ig VJ genes using the IMGT database, as for Table 1. Clone numbers designate phagemid clone containing Lc (GKK/GKL#). Sequences are grouped by common V gene usage. Clonal groups, as identified by common VJ germ-line gene usage and junctional mutation, are boxed.

Clonal group	Clone	V gene	J gene	
Kappa	1	GKK 15 LC	IGKV3-20*01	IGKJ1*01
		GKK 69 LC	IGKV3-20*01	IGKJ1*01
		GKK 1 LC	IGKV3-20*01	IGKJ1*01
	2	GKK129LC <sup>a</sup>	IGKV3-20*01	IGKJ2*01
		GKK 61 LC	IGKV3-20*01	IGKJ5*01
		GKK 14LC <sup>a</sup>	IGKV1D-39*01	IGKJ1*01
		GKK 88 LC	IGKV1D-39*01	IGKJ1*01
		GKK 16LC <sup>a</sup>	IGKV2D-28*01	IGKJ1*01
		GKK125 LC	IGKV2D-28*01	IGKJ1*01
		GKK137LC <sup>a</sup>	IGKV4-1*01	IGKJ3*01
		GKK 86LC <sup>a</sup>	IGKV2-30*01	IGKJ1*01
		GKK118 LC	IGKV3-11*01	IGKJ1*01
		GKK139 LC	IGKV3-11*01	IGKJ4*01
		GKK 22 LC	IGKV1-16*01	IGKJ1*01
		Lambda	1	GKL 11 LC
GKL 20 LC	IGLV1-51*01			IGLJ3*01
GKL 26 LC	IGLV1-51*01			IGLJ3*01
GKL 27 LC	IGLV1-51*01			IGLJ3*01
GKL 34 LC	IGLV1-51*01			IGLJ3*01
GKL 55 LC	IGLV1-51*01			IGLJ3*01
GKL 61 LC	IGLV1-51*01			IGLJ3*01
GKL 64 LC	IGLV1-51*01			IGLJ3*01
GKL 3 LC	IGLV1-51*01			IGLJ3*01
GKL 18 LC	IGLV1-51*01			IGLJ3*01
2	GKL 35 LC		IGLV2-14*01	IGLJ3*01
	GKL 41 LC		IGLV2-14*01	IGLJ3*01
	GKL 4 LC		IGLV2-14*01	IGLJ3*01
	GKL 10 LC		IGLV3-19*01	IGLJ3*01
	GKL 38 LC		IGLV1-40*01	IGLJ3*01

<sup>a</sup> Members of clonal groups with SC library members.

Table 3 *Germ-line Hc V gene usage and clonal groups of patient 2*  
Hc sequences for the PT combinatorial libraries were analyzed as in Table 1.

Clonal group	Clone	V gene	D gene	J gene
1	PTL 36 HC	IGHV1-69*01	IGHD1-20*01	IGHJ5*02
	PTL 101 HC	IGHV1-69*01	IGHD1-20*01	IGHJ5*02
	PTL 106 HC	IGHV1-69*01	IGHD1-20*01	IGHJ5*02
	PTL 116 HC	IGHV1-69*01	IGHD1-20*01	IGHJ5*02
	PTL 118 HC	IGHV1-69*01	IGHD1-20*01	IGHJ5*02
	PTK 7 HC	IGHV1-69*01	IGHD1-20*01	IGHJ5*02
	PTK 21 HC	IGHV1-69*01	IGHD1-20*01	IGHJ5*02
	PTL 111 HC	IGHV1-69*01	IGHD1-20*01	IGHJ5*02
	PTK 2 HC	IGHV1-69*01	IGHD1-20*01	IGHJ5*02
	PTK 17 HC	IGHV1-69*01	IGHD1-20*01	IGHJ5*02
	PTL 105 HC	IGHV1-69*01	IGHD1-20*01	IGHJ5*02
	PTL 126 HC	IGHV1-69*01	IGHD3-10*01	IGHJ5*02
2	PTL 32 HC	IGHV4-59*01	IGHD3-9*01	IGHJ3*02
	PTL 103 HC	IGHV4-59*01	IGHD3-9*01	IGHJ3*02
	PTL 121 HC	IGHV4-59*01	IGHD3-9*01	IGHJ3*02
	PTK 4 HC	IGHV4-59*01	IGHD3-9*01	IGHJ3*02
	PTK 19 HC	IGHV4-59*01	IGHD3-9*01	IGHJ3*02
3	PTL 108 HC	IGHV4-59*01	IGHD4-11*01/inv	IGHJ3*02
	PTK 22 HC	IGHV4-59*01	IGHD4-11*01/inv	IGHJ3*02
4	PTK 1 HC	IGHV3-30*01	IGHD1-20*01/inv	IGHJ4*02
	PTK 12 HC	IGHV3-30*01	IGHD1-20*01/inv	IGHJ4*02
	PTK 15 HC	IGHV3-30*01	IGHD1-20*01/inv	IGHJ4*02
	PTL 122 HC	IGHV3-30*01	IGHD6-19*01	IGHJ4*02
	PTL 130 HC	IGHV1-3*01	IGHD4-17*01	IGHJ3*02
	PTK 9 HC	IGHV3-66*01	IGHD3-9*01	IGHJ3*02
	PTL 123 HC	IGHV3-74*02	IGHD3-10*01	IGHJ4*02
	PTL 119 HC	IGHV4-30-2*04	IGHD4-17*01	IGHJ4*02
	PTK 18 HC	IGHV4-b*01	IGHD7-27*01	IGHJ4*02
	PTK 10 HC	IGHV3-11*01	IGHD6-19*01	IGHJ4*02

Table 4 *Germ-line Lc V gene usage and clonal groups of patient 2*  
Lc sequences for the PT combinatorial libraries were analyzed as in Table 2.

Clonal group	Clone	V gene	J gene	
Kappa	1	PTK15LC	IGKV1D-39*01	IGKJ1*01
		PTK17LC	IGKV1D-39*01	IGKJ1*01
		PTK18LC	IGKV1D-39*01	IGKJ1*01
		PTK19LC	IGKV1D-39*01	IGKJ1*01
		PTK10LC	IGKV1D-39*01	IGKJ2*01
	2	PTK 1LC	IGKV4-1*01	IGKJ5*01
		PTK 4LC	IGKV4-1*01	IGKJ5*01
		PTK22LC	IGKV4-1*01	IGKJ1*01
	3	PTK11LC	IGKV2D-28*01	IGKJ1*01
		PTK21LC	IGKV2D-28*01	IGKJ4*01
		PTK 9LC	IGKV1-9*01	IGKJ4*01
		PTK 2LC	IGKV1-17*01	IGKJ2*01
		PTK12LC	IGKV3-11*02	IGKJ4*01
		PTK 7LC	IGKV3-20*01	IGKJ2*01
	Lambda	1	PTL 101 LC	IGLV6-57*01
PTL 103 LC			IGLV6-57*01	IGLJ3*01
PTL 111 LC			IGLV6-57*01	IGLJ3*01
PTL 123 LC			IGLV6-57*01	IGLJ3*01
PTL 130 LC			IGLV6-57*01	IGLJ3*01
PTL 118 LC			IGLV6-57*01	IGLJ3*01
2		PTL 105 LC	IGLV4-69*01	IGLJ3*01
		PTL 116 LC	IGLV4-69*01	IGLJ3*01
		PTL 126 LC	IGLV4-69*01	IGLJ3*01
3		PTL 32 LC	IGLV2-23*01	IGLJ3*01
		PTL 106 LC	IGLV2-23*01	IGLJ3*01
		PTL 36 LC	IGLV7-43*01	IGLJ3*01
		PTL 122 LC	IGLV7-43*01	IGLJ3*01
		PTL 121 LC	IGLV7-46*02	IGLJ3*01
		PTL 108 LC	IGLV1-51*01	IGLJ3*01
PTL 119 LC	IGLV3-19*01	IGLJ3*01		

For patient 1 (GK), a striking degree of clonality was revealed in Hc and Lc libraries. Among the VH sequences, there were six clonal groups, ranging in size from two to six members, accounting for 17/30 (57%) sequences examined (Table 1). Reuse of the same V and J with different D regions was evident with VH groups 1, 2, and 3 with

nonreiterated clones and between groups 4 and 5 reiterated clones, in which the different D regions (and junctional mutations) signal independent, nonisoclonal B-cell origins. Among the  $\kappa$  sequences, there were two clonal groups consisting of 2 and 3 members, accounting for 5/15 (33%)  $\kappa$  Lcs examined (Table 2). Among the  $\lambda$  sequences, there were two clonal groups, consisting of 2 and 8 members, accounting for 10/15 (67%) total  $\lambda$  sequences analyzed.

A marked clonality was also evident in the libraries from patient 2 (PT). Within the 29 usable VH sequences, four clonal groups accounted for 21 (69%) sequences examined (Table 3). Clonal groups consisted of 2–9 members. As seen in patient 1, there was a similar reuse of specific VH genes with different D and/or J by different progenitor B cells, with groups 1 and 4 sharing VH with nonreiterated clones and groups 2 and 3 sharing with each other. Among the PT  $\kappa$  sequences, there were three clonal groups consisting of 2–4 members each, accounting for 6/14 (42%) of the sample (Table 4). Among the  $\lambda$  sequences, there were three clonal groups consisting of 2–5 members, accounting for 9/16 (62%) of total  $\lambda$  sequences analyzed.

Table 5 summarizes these findings. In comparing the immune reactions of the two patients, one VL is shared (V $\lambda$  V2.1), but the response is otherwise distinct between them. The use of different portions of the immune repertoire by different individuals with the same immunogen is typical (e.g., Ref. 36). The oligoclonality is very similar between the patients, with 6 clones (patient 1) and 4 clones (patient #2) dominating the  $\gamma$ 1 response by the VH analyses. Together, these analyses show a remarkable degree of repertoire focusing in MC tumor tissue, with 30–70% of the isolates being repeated within these small samples, suggesting that a relatively few original B-cell clones (4–6) account for  $\geq$ 50% of the total repertoire in the tumors.

Finally, the meaning of these statistics warrants clarification. The range of 30–70% pertains only to the fraction of the library that is comprised by these specific 4–6 clones and says nothing about those sample members that are represented only once. It is quite plausible that most or all of those IgV regions present singly ( $n = 1$ ) in the sample are also elicited by the same neoantigen(s) as the more abundant clones, but the single clones are present merely in the tumor at an average lower frequency that represents them only once in these specific samples. Thus, this estimate of a focused repertoire may be viewed as a minimum estimate that would be likely to be fractionally increased with a larger sample size.

Table 5 *Repertoire focus in MC*

V genes reiterated ( $n > 1$ ) in combinatorial library samples, with confidence limits (c.l.) on true fraction in library for these clones. Related clones were defined as having the same V(D)J and sharing N/P junctional mutations versus germ line. V5-a\*01 in patient 1 and V4-59\*01 in patient 2 were each reiterated with different D and/or J pairs, with different junctional sequences, indicating different original progenitor B cells.

Patient #	VH	V $\kappa$	V $\lambda$	
1	V5-51*01 (6)	V3-20*01 (3)	V1-51*01 (8)	
	V5-51*03 (2)	V2D-28*01 (2)	V2-14*01 (2)	
	V5-a*01 (3)			
	V5-a*01 (2)			
	V1-18*01 (2)			
	V1-2*02 (2)			
	Fraction of library	17/30	5/15	10/15
	Percentage	57%	33%	67%
	95% c.l.	37–75%	12–62%	38–88%
	2	V1-69*01 (9)	V1D-39*01 (4)	V6-57*01 (5)
V4-59*01 (5)		V4-1*01 (2)	V4-69*01 (3)	
V4-59*01 (2)		V2D-28*01 (2)	V2-23*01 (2)	
V3-30*01 (3)				
Fraction of library		19/29	8/14	10/16
Percentage reiterated		66%	57%	63%
95% c.l.		47–83%	32–84%	34–86%

c.l. = confidence limits.

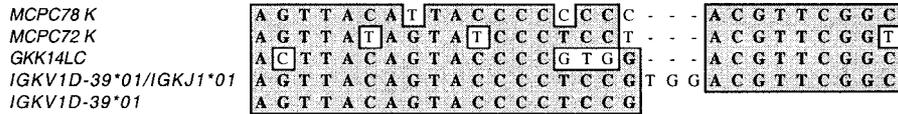


Fig. 2. Clonality determination by common V(D)J germ-line gene usage and somatic and junctional mutations. Two clonal sequences (GKK14LC and MCPC66K) are aligned with nonclonal sequences with shared germ-line gene usage (MCPC72 and MCPC78) and with cognate germ-line VJ and V genes to indicate position of junctions. Identity is shown by shading. Gaps are the result of exonuclease activity, whereas nongermline nucleotides are junctional mutations.

“Antigen-competent” VH+VL Pairing: Phage Panning

Whereas the foregoing confirmed an IgG-dominant PC response and a focused immune repertoire, the ultimate proof of an antigen-driven response is documented reactivity with an antigen. In the present instance, this assessment is complicated by the fact that the eliciting antigen is unknown. Thus, we sought to test for antigen and tissue reactivity by panning procedures with the libraries for which the phage Fab has specific advantages.

Because the libraries are randomized for VH+VL for each Fab combination, the original VH-VL pairing in the tumor plasma cells is unknown. With 6 VH and 6 VL groups equally represented (neglecting mutations), there is a possibility of  $6 \times 6 = 36$  combinations, and the chance of any given pair being correct is  $6/36 = 1/6$ . When the 6 groups are diluted by additional VH and VL, the chance of randomly selecting an original group pair is proportionately less, to the second power. If the antigen is known, it is possible to “pan” the libraries against antigen with selection of reactive clones from which the original cognate pairing might be inferred, or at least “compatible” VH+VL pairs may be identified. Because of the phenomenon of Lc promiscuity in which different Lcs may pair with an antigen-specific Hc and maintain antigen recognition (32), this latter possibility could generate productive but nonoriginal pairings.

To examine the possibility that the eliciting antigen is a common breast cancer-associated antigen, we examined two of the best characterized.

**Her2/neu.** Pupa *et al.* (37) showed Her2/neu reactivity of EBV-transformed patient peripheral B cells when tumors of the patients (a) overexpressed Her2/neu; and (b) were infiltrated. It is noted, however, that in four of four MC tumors tested, there was no Her2 overexpression (Ref. 38),<sup>5</sup> and it was, therefore, less likely that Her2/neu is the principal eliciting antigen that gives MC its characteristic PC infiltrates. Nevertheless, we tested this directly. Using purified recombinant Her2/neu<sup>ECD</sup> protein (39), we adapted our phage Fab ELISA (21) to detect specificity for this antigen (see “Materials and Methods”). We panned the phage Fab libraries from the two MC patients (Fig. 3) and individually tested the 60 random phage Fab clones of Tables 1–4 (not shown). No Her2/neu reactivity was detected.

**p53.** A portion of breast carcinomas with mutated p53 may have serum antibodies to p53 but against nonmutated regions of the protein (40, 41). No data specifically link anti-p53 responses to PC infiltration in MC or NOS tumors. Nevertheless, we tested this directly using purified glutathione S-transferase-p53 fusion protein as above. No p53 reactivity was detected in any of the libraries in 4 cycles of panning (Fig. 3) or in testing of the 60 single clones (not shown).

Under the conditions of these panning assays, if  $1:10^6$  phage Fab had been reactive with either of these antigens, the panning enrichment of specific phage would have been readily detected by these methods, as demonstrated previously by us (21) and others (30, 31, 42). These negative data effectively rule out these two prominent breast carcinoma-associated oncogenes as the eliciting neoantigen in these PC-infiltrated MC tumors.

On the chance that the unknown antigen was a surface-expressed

protein, we devised a cell-based panning method to select reactive phage Fab (29). Our positive control tests against a moderately expressed cell-bound antigen (~5–10,000/cell) showed enrichment of specific phage Fab by this technique at ~25-fold per round of panning (29), which would have yielded an ~100% pure population after 6 rounds with  $1:10^6$  specific-to-nonspecific phage. Similarly, cloned specific phage Fab were effective in staining procedures and in detecting cellular antigen by flow cytometry (29). For the present tests, an MC cell line (HTB-24) and a negative-control cell line (293) were used in 6 rounds of panning, but no repertoire shift or titer enrichment was detected with the unselected libraries of either patient (not shown). Results were also negative by flow cytometry in direct binding assays of the 60 phage-Fab clones against tumor cells (not shown). These negative cell-panning results forced a conclusion that the antigen: (a) was not surface-expressed; (b) was surface-expressed at too low a level for panning enrichment or for flow cytometry detection against background; or (c) was lost from the tumor cell line. No adequate source of MC tumor was available to employ primary tissue for panning, and viability after disaggregation was poor.

Thus, we were unable to identify either cognate VH-VL pairs or the eliciting antigen(s), or even to confirm the localization of eliciting antigen to the membrane or within the cell. Additional efforts to identify the antigen were deferred until native VH-VL pairs could be definitively established, thus enabling traditional molecular and biochemical methods for retrieving the antigen.

“Native” VH+VL Pairing: SC RT-PCR

Having failed to select for cognate VH+VL pairs independent of knowledge of the immunizing antigen, it proved necessary to identify the pairs within individual PCs. Accordingly, in parallel with the above panning efforts, we undertook development of methods for

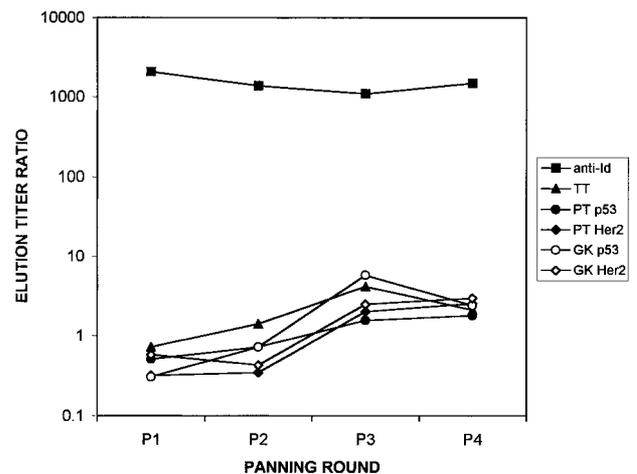


Fig. 3. Demonstration of library nonreactivity with p53 and Her2/neu. Wells coated with p53, Her2/neu, or MAT as antigens. Panning was done with phage Fab: MC libraries PT and GK (pool  $\gamma$ ,  $\kappa + \gamma$ ,  $\lambda$ ) against p53 or Her2/neu; and antitetanus toxoid (negative control) and anti-Id (positive control) against MAT (20). Ratio of eluted:input titers shown. After four rounds of panning (P1–P4), only positive control anti-Id phage Fab showed specific binding to antigen and enrichment with 100–1000-fold advantage in titers.

<sup>5</sup> M. Press, personal communication.

Table 6 Germ-line V gene usage and clonal groups of patient 1 single PCs

Clone numbers indicate well of plate containing amplified IgG1 V genes (MCPC#). Hc and Lc sequences for the single PCs were analyzed as in Tables 1 and 2. Clonal groups, as identified by common V(D)J germ-line gene usage and junctional mutation, are boxed.

SC clone	Hc			Lc	
	V gene	D gene	J gene	V Gene	J gene
MCPC01	IGHV3-9*01	IGHD2-21*02	IGHJ4*02	IGKV3-20*01	IGKJ3*01
MCPC09	IGHV3-21*02	IGHD6-19*01	IGHJ5*02	IGKV1-27*01	IGKJ4*01
MCPC15	IGHV3-21*02	IGHD6-19*01	IGHJ5*02	IGKV1-27*01	IGKJ4*01
MCPC14	IGHV1-18*01	IGHD6-13*01	IGHJ4*02	IGKV1-5*03	IGKJ2*01
MCPC23 <sup>a</sup>	IGHV3-73*01	IGHD5-24*01	IGHJ4*03	IGKV4-1*01	IGKJ3*01
MCPC34 <sup>a</sup>	IGHV1-18*01	IGHD3-16*01	IGHJ6*03	IGKV2D-28*01	IGKJ1*01
MCPC38	IGHV3-21*02	IGHD2-2*03/inv	IGHJ5*02	IGKV1-17*01	IGKJ4*01
MCPC44	IGHV1-2*02	IGHD3-16*01	IGHJ4*03	IGKV2D-29*01	IGKJ1*01
MCPC51 <sup>b</sup>	IGHV1-18*01	IGHD6-19*01	IGHJ4*02	IGKV1-5*03	IGKJ1*01
MCPC54	IGHV3-23*01	IGHD3-10*02/inv	IGHJ1*01	IGLV3-10*01	IGLJ7*02
MCPC57	IGHV2-5*09	IGHD5-12*01	IGHJ5*02	IGLV3-21*02	IGLJ4*01
MCPC61	IGHV1-46*03	IGHD3-16*03	IGHJ6*03	IGKV1-9*01	IGKJ4*01
MCPC66 <sup>a</sup>	IGHV4-4*01	IGHD3-16*01	IGHJ6*03	IGKV1D-39*01	IGKJ1*01
MCPC71 <sup>a</sup>	IGHV3-30*03	IGHD2-21*02	IGHJ4*03	IGKV2-30*01	IGKJ1*01
MCPC72 <sup>b</sup>	IGHV4-62*02	IGHD6-25*01	IGHJ6*02	IGKV1D-39*01	IGKJ1*01
MCPC75	IGHV3-9*01	IGHD7-27*01	IGHJ5*02	IGKV1-9*01	IGKJ1*01
MCPC78	IGHV4-61*02	IGHD3-22*01	IGHJ5*02	IGKV1D-39*01	IGKJ1*01
MCPC85 <sup>a</sup>	NA	NA	NA	IGKV3-20*01	IGKJ2*01

<sup>a</sup> Members in clonal groups with combinatorial library Lc genes.  
<sup>b</sup> Members in clonal groups with combinatorial library Hc genes.

isolation of single PCs and recovery of VH+VL sequences by SC RT-PCR, which had not been standardized previously for human Ig genes (23). (An equivalent method has since appeared in the literature; Ref. 43.) This method was applied to prepare a SC IgG library with authentic VH-VL pairings.

PCs were isolated from disaggregated tumor from patient 1 and amplified by RT-PCR. Of 90 wells, one Lc ( $\kappa$  or  $\lambda$ , but not both) amplified from 28 and IgG1 Hc amplified from 20. One well of the 90 had both  $\kappa$  and  $\lambda$  products, implying two cells in a well, and was excluded from the analysis. All 20 of the IgG Hc amplifications had successful Lc amplifications, of which 18 were  $\kappa$  and 2 were  $\lambda$ , indicating a preponderance of  $\kappa$ -positive plasma cells. Useable sequence was obtained from 18. Of the 18, 1 (MCPC85) had useable sequence for Lc only.

Of the total PCs identified by Lc amplification, 71% (20/28) were IgG1. The remaining 29% of PCs (8/28) with Lc and no Hc amplification indicate an isotype other than IgG1, meaning that the total of IgA, IgM, and IgG2-4 PCs account for <30% of this tumor PC sample. No cells showed amplification of Hc without Lcs, whereas positive controls for Hcs did amplify, indicating that lack of Hc amplification in Lc-only wells is almost certainly attributable to a non-IgG1 Hc rather than PCR failure. Because the SC library is an unbiased sampling of the tumor, this 71% fraction (52–86%; 95% confidence interval) estimates the proportion of the total antibody response that is IgG1 and implies that this isotype is a fair sample of the total response that is present. This fraction may be compared with the enumeration of PCs in this tumor by immunohistochemistry (Fig. 1) in which ~70% of cells were IgG+ (207/290) versus ~30% for IgA+ and IgM+ combined.

The marked  $\kappa$  predominance *in vivo* (16/18; 91%) among the IgG1+ samples was unexpected and significantly different from the normal fraction of 60:40  $\kappa$ : $\lambda$  ( $P < 0.01$  by  $\chi^2$ ; Ref. 44), whereas only 3/8 (38%) of the non-IgG1 PCs in the sample were  $\kappa$ . In contrast, a control sample of 13 random tonsil PCs selected and analyzed identically consisted of 8  $\kappa$  (62%) and 5  $\lambda$  sequences (38%), reflecting typical *in vivo* ratios (23). Immunohistochemistry of patient 1 tumor tissue indicated a slightly higher level of  $\lambda^+$  than  $\kappa^+$  PCs (not shown). Because the SC RT-PCR procedure samples the entire disaggregated tumor section but the immunohistochemical analysis samples only a single location, the divergent  $\kappa$ : $\lambda$  tissue staining results may reflect

heterogeneity within the tumor. We are unaware of a particular significance of a  $\kappa$ -dominated immune response.

Table 6 provides a clonal analysis of the individual Hc and Lc sequences in the SC library. Only two PCs (MCPC9 and MCPC15) were clonally related within this sample. However, when considered together with the combinatorial library sample (Table 7), an additional 7 members of the SC library were in clonal groups (total 9/20; 45%), 5 derived from comparison with the  $V\kappa$  clones (Table 2) and 2 with the VH clones (Table 1). Similarly, the SC library revealed that 1 additional VH member and 4 additional  $V\kappa$  members of the combinatorial library were clonal. Neither of the  $\lambda^+$  SC clones overlapped with any VH or VL of the combinatorial library sample.

Considered together, the combinatorial and SC library from patient 1 indicate a total of 8 VH clonal groups, accounting for 22/45 or 49%

Table 7 Clonal overlap of combinatorial and SC libraries

The presence of clonally related sequences between the GK combinatorial (combin) and single cell (SC) libraries is shown, with clonal groups as defined in Tables 1–4 and additional groups as needed to incorporate the SC library contribution.

Antibody	Group	Source		Total	P( $\chi^2$ )
		Combin	SC		
V $\gamma$	1	6	0	6	0.04
	2	2	0	2	
	3	2	1	3	
	4	3	0	3	
	5	2	0	2	
	6	2	0	2	
	7	1	1	2	
	8	0	2	2	
	NR <sup>a</sup>	10	13	23	
	Total	28	17	45	
V $\kappa$	1	3	0	3	0.49
	2	2	1	3	
	3	1	1	2	
	4	1	1	3	
	5	1	1	2	
	6	1	1	2	
	7	0	2	2	
	NR <sup>a</sup>	5	9	14	
Total	14	16	30		
V $\lambda$	1	8	0	8	0.20
	2	2	0	2	
	NR <sup>a</sup>	5	2	7	
	Total	15	2	17	

<sup>a</sup> NR, not reiterated in the sample.

of Hc sequences, 7 V $\kappa$  clonal groups, accounting for 16/30 or 53% of  $\kappa$  Lcs, and 2 V $\lambda$  clonal groups, accounting for 10/17 or 59% of the  $\lambda$  Lcs. These results are compatible with ~8 progenitor B-cell clones accounting for 50% of the response in the tumor of this patient.

Interestingly, none of the VH+VL of the IgG, $\kappa$  or the IgG, $\lambda$  members represented in the GK SC library was appropriately paired by chance in the GK combinatorial library, confirming our estimation (above) of a low probability of finding an original pairing in this small combinatorial sample. Similarly, although ~50% of the SC library was reiterated when compared with the phage Fab library, only one sequence was reiterated ( $n = 2$ ) within the sample of 19 single PCs, and this clone was not present among either the VH or VL libraries of the phage Fab. The combined  $\kappa$  samples have reiteration in 16/30 clones or 53% with a mean frequency for all of the reiterated clones of  $0.08 \pm 0.02$  (SD). With 7 clones of 0.08 frequency, the most probable number of reiterated clones in a sample of 16 single ( $\kappa$ ) PCs is just 1, consistent with our observations (Table 6; calculation not shown). These observations emphasize the importance of adequate sample sizes to demonstrate reiteration when the frequencies of individual clones are in the range of  $\leq 10\%$  in which a larger SC library would have been required to demonstrate focus if it were the sole source of representation.

Statistical analysis showed good concordance between the V $\kappa$  of the combinatorial and SC libraries ( $\chi^2$ ;  $P = 0.49$ ) in which the SC library was  $\gamma 1$  Hc derived. This is a particularly important point, because it indicates that the commitment to use the  $\gamma 1$  specific Hc primer in the combinatorial library was appropriate even if the cognate VH+VL pairing was unknown, in effect confirming by a separate measure the IgG1 dominance of the immune response in the tumor of this patient. Although clonality is evident in the  $\lambda$  repertoire from the combinatorial library, it is a minor part of the total intratumoral response, and the small sample size of the SC library does not allow a suitably powered comparison ( $P = 0.20$ ). Comparison of the VH, however, showed a significantly divergent representation be-

tween the two library formats ( $P = 0.04$ ). The most abundant clonal group in the combinatorial library (1; Table 1) was absent in the SC library. This could suggest an amplification bias favoring the V5 family in preparing the phage Fab library in comparison with the SC library, which cannot be biased. The absence of repeated V5 family clones in the phage Fab library of tumor 2 (Table 3) might have been cited to counter the suggestion of a V5 bias in the PCR, but the PT combinatorial library (like the GK SC library) was prepared with a mixture of VH family primers rather than the single VH consensus primer applied in the GK library, and is, thus, uninformative to this point. The Lc GK libraries were prepared with mixtures of family-homologous primers and showed excellent concordance between the combinatorial and SC libraries. Finally, it is possible that the discrepancy between the GK SC and combinatorial VH libraries is attributable to small numbers and that a larger SC library would have revealed greater convergence with the combinatorial library.

The overlap between SC and combinatorial library sequences additionally confirm the existence of a focused intratumoral antibody repertoire.

**Somatic Mutation of Ig V Genes**

Somatic mutations of Ig V(D)J genes occur in B cells after antigen contact and are typically concentrated in the antigen-contacting CDR regions in affinity-matured Ig. Levels and patterns of mutation within the IgV regions (FR1-CDR1-FR2-CDR2-FR3) of MC-infiltrating PCs were analyzed. CDR3 was excluded because of the high incidence of deletions and D gene inversions in this region that complicate the analysis. The somatic mutation pattern *versus* germ line is shown in Fig. 4A for the largest VH clonal group. In this instance, it was possible to trace a common progenitor B cell and branching order through at least four rounds of division and differentiation, leading to three "clades" within this clonal group (Fig. 4B).

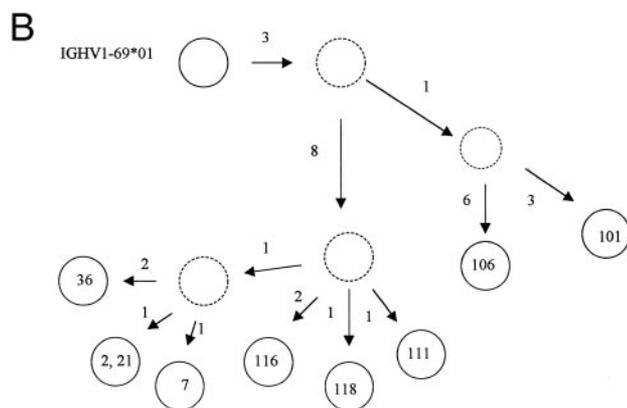
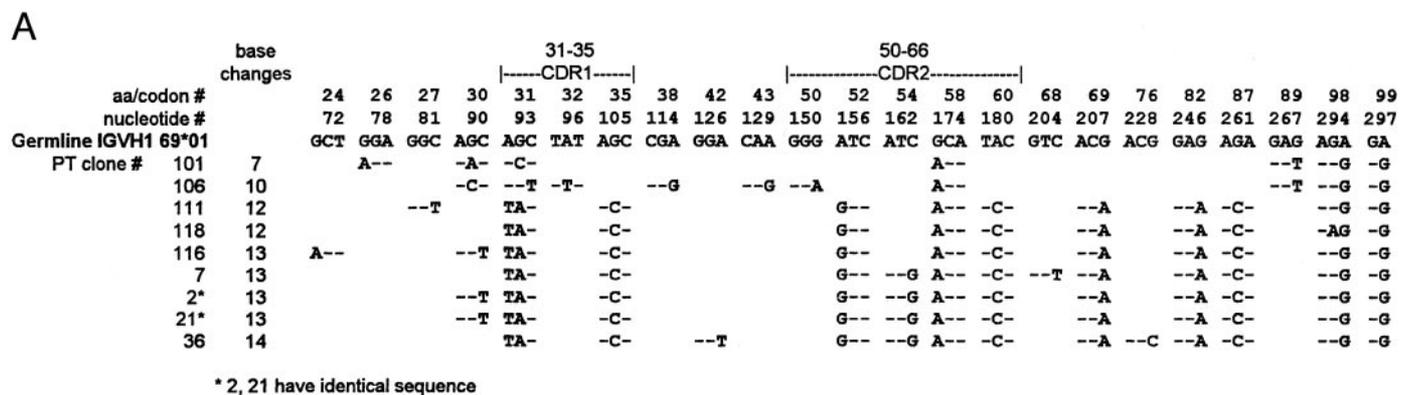


Fig. 4. Somatic mutation diversification in clonal group of tumor-infiltrating PCs. VH clonal group 1 of patient 2 was derived from a common progenitor B cell that used the IGHV1-69\*01 gene (Table 3). A, alignment of VH sequences of clonal group. Alignment begins at base 23 of FR1 to exclude the primer annealing regions. Only codons containing mutations from germ line are shown. Clones 2 and 21 have the same sequence but are nonidentical clones, having been amplified with different primers (VH1a and VH6a, respectively (15)). B, clade analysis with genetic tree. Branching order of B and/or PC clones. Solid line circles (○) indicate germ line and actual sequenced PC clones. Dotted circles indicate deduced intermediates. Numbers within circles indicate designation of cloned gene. Numbers next to arrows indicate number of V gene mutations at each step.

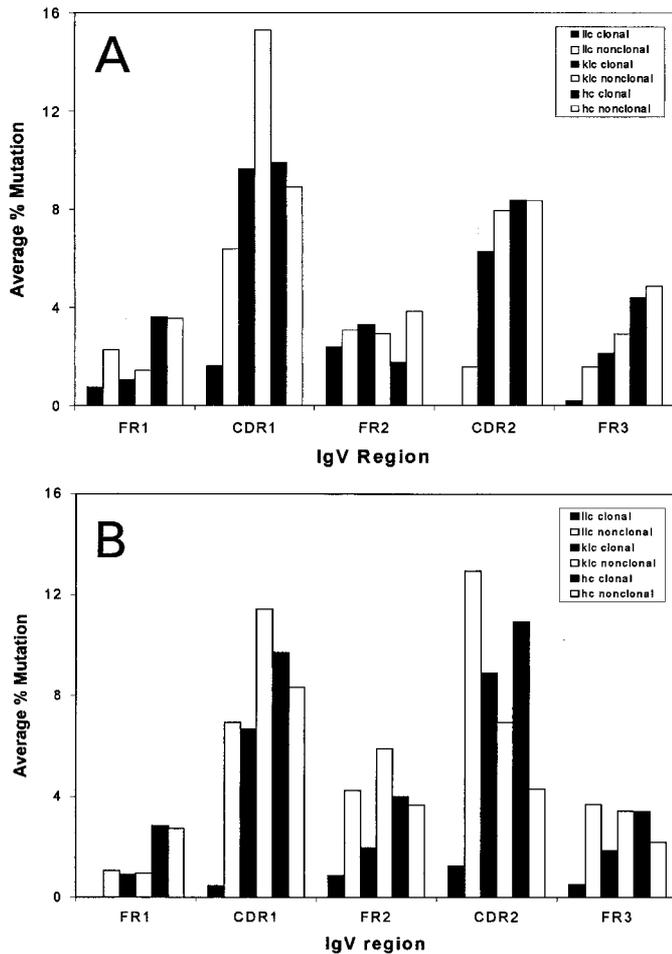


Fig. 5. Concentration of somatic mutations in CDRs. Percentage mutation of IgV genes from germ-line IgV gene. Percentage mutation for individual regions (FR1, CDR1, FR2, CDR2, and FR3) was calculated as the number of changes divided by the total number of nucleotides for the region. There was no systematic difference in the distribution of mutations between clones reiterated ( $n > 1$ ; clonal; ■) and those present only once (nonclonal; □) in the sample. Average percentage mutation was calculated (from left to right) for clonal  $\lambda$  Lcs, *llc clonal*; nonclonal  $\lambda$  Lcs, *llc nonclonal*; clonal  $\kappa$  Lcs, *klc clonal*; nonclonal  $\kappa$  Lcs, *klc nonclonal*; clonal Hcs, *hc clonal*; and nonclonal Hcs, *hc nonclonal*. A, patient 1. B, patient 2. Patient 1 data include both combinatorial and SC library sequences.

The mutation distributions of patient 1 (combinatorial and SC libraries) and patient 2 (combinatorial libraries) IgV genes versus germ line are shown in Fig. 5. Sequences were grouped for analysis on the basis of belonging or not belonging to a clonal group, as defined in Tables 1–4 and 6. Average values were determined for each group. Whereas overall group average levels of mutation in comparison to cognate germline V(D)J Ig genes are between 1 and 6%, total mutation levels ranged as high as 10% in individual sequences.

**Patient 1.** As seen in Fig. 5A, there is a higher level of mutation within the CDR regions of all of the patient 1 IgV regions compared to framework regions (FR), with the exception of  $\lambda$  light chains. For example, the average percentage of mutation of patient 1 clonal  $\kappa$  light chains across the Ig V region is:

Table 8 Example of V region mutation distribution

	FR1	CDR1	FR2	CDR2	FR3
klc clonal	1.0	9.6	3.3	6.3	2.1

Therefore, although the overall level of mutation for this group is only 2.8%, higher concentrations of mutations are clustered within the CDRs.

In contrast, clonal  $\lambda$  Lcs are almost completely unmutated, with overall mutation levels of 0.9%, including the two  $\lambda+$  single PCs from the pool of 20 analyzed (MCPC54 and 57), with only 4 mutations (0.9%) each in the entire Ig V region. Nonclonal  $\lambda$  Lcs have overall levels of mutation only slightly lower than in the other non- $\lambda$  groups at 2.5%, but mutations are not clustered within the CDRs.

**Patient 2.** As seen for patient 1, there is similarly a marked clustering of mutations within the CDRs in patient 2 in all of the sequences except clonal  $\lambda$  Lcs (Fig. 5B), which are, again, nearly unmutated. Unlike patient 1, however, patient 2 nonclonal  $\lambda$  chains have increased somatic mutation within the CDR regions.

In summary, mutations cluster within the CDR regions, a finding that is consistent with somatic mutation and affinity maturation of these sequences, and mutation frequencies do not differ appreciably between the “clonal” and “nonclonal” groups. This is not surprising in that the nonclonal groups are probably, in fact, mostly clonal but reiterated in the unselected libraries at a lower average frequency than those clones that are represented more than once. As stated above, the estimation of clonal fraction in the tumors is a lower estimate. Overall mutation levels and patterns are consistent with published values for other protein antigen-selected human antibodies (45).

**DISCUSSION**

Plasmacytic infiltration is a diagnostic feature of MC of the breast and is present in an equal number but smaller proportion of the more abundant NOS infiltrating ductal carcinomas. The objective of this study was to determine if these PC infiltrates represent a tumor-specific humoral immune response as opposed to a nonspecific cytokine-driven recruitment. Characteristics of a local antigen-driven B-cell response include a highly restricted Ig repertoire, IgG isotype, somatic mutation of Ig, and PC differentiation (46). Local secretion of chemotactic agents such as interleukin-6 could attract peripheral blood B cells in an antigen-independent manner that are largely unrestricted, IgM+, and unmutated (47, 48).

For the present study, we examined the IgV sequences of MC tumor-infiltrating PCs for similar germ-line gene usage to determine proliferation (clonality) and for evidence of antigen dependence such as somatic mutation within the CDRs and IgG isotype. In a recent evaluation, Kotlan *et al.* (49) performed RT-PCR amplification of total VL and IgG VH from one MC tumor and sequenced several random isolates: 9 VH $\gamma$ , 5 V $\kappa$ , and 10 V $\lambda$ . They offered a tentative conclusion of an oligoclonal Ig amplification based on an apparent reiteration of clones even within this limited sample. By the more extensive examination of the present study, we have made a definitive conclusion of local B proliferation compatible with local antigen exposure as determined by a high degree of clonality, IgG isotype, and somatic mutation in intratumoral PCs. In the absence of a defined tumor antigen, it was not possible to specify appropriately reactive VH+VL pairs from our combinatorial libraries. Similarly, the failure to enrich for phage-Fab clones on tumor cell panning suggested that the antigen is on the surface at too low a level to allow selection by this method, is not a membrane protein, or has been lost from the MC cell line we used. The development and application of single PC RT-PCR techniques in our study validated the repertoire focus of our initial combinatorial cloning effort and, furthermore, yielded appropriately paired VH+VL antibody fragments that will be important to future antigen identification efforts.

**Isotype Restriction of MC-infiltrating PCs.** As determined by both immunohistochemistry and SC RT-PCR, we confirmed that the infiltrating PCs of MC are predominantly (~70%) IgG+ rather than IgM or IgA. IgM is generally seen in primary tissue immune responses, whereas IgA predominates in all of the normal secretory

epithelium. The IgG-positive response observed in this and other studies of MC and in other PC-infiltrated NOS breast tumors (11) is consistent with early inferences of an intratumoral protein antigen and a matured immune response (10, 11). That the eliciting antigen is tumor-specific rather than broadly expressed is reinforced by the observation that the PC infiltrates in these tumors do not extend into the adjacent normal breast tissue (3–5, 8). Approximately 71% (20/28) of single PCs were IgG1+ in the tumor of the one patient in whom it was directly tested. IgG1 is the predominant antibody response in T-dependent antigens (47), supporting a presumption of a protein origin for the antigen.

#### Restricted Immunoglobulin Repertoire of MC-infiltrating PCs.

It has been estimated that a specific B-cell clone occurs in the peripheral blood at a frequency of no more than 1/20,000 (50). Even in hyperimmunized individuals, antitetanus antibodies were present in a total B-cell library in only 1:1000 to 1:5000 clones, and only 2/8 reactive clones that were sequenced showed the same V gene usage (39, 51). Similarly, in a limited sample of 13 single-tonsil PCs examined by RT-PCR, no reiteration was observed (23), consistent with a typically diverse representation in tonsil (26).

Clonal groups in the present study were defined by shared germ-line gene usage and shared junctional diversity patterns, because these recombination events occur in the bone marrow before circulation into the periphery. As determined by analysis of IgG sequences from both the combinatorial and SC libraries, the IgV gene repertoire used by tumor-infiltrating PCs was highly restricted in both patients, with 4–8 progenitor B cells accounting for 40–80% of the total response. The focused representation within the combinatorial library from patient 1 and by implication patient 2 was validated by the overlap of sequences from the SC library. Sequencing from single tumor-infiltrating PCs provides a sampling method that is not subject to either cloning-induced or PCR bias. Our results, therefore, confirm an antigen-selected response within the tumor and rule out a nonspecific cytokine-induced origin for the lymphoplasmacytic infiltrates.

There is broad V gene family representation in response to both exogenous and self antigens, although bias toward certain families has been suggested (45, 52). Within the GK SC library, Hc and Lc representation corresponded approximately to numerical representation of germ-line genes within the genome. For example, the largest IGHV family, IGHV3, contains 46% of all genomic germ-line IGHV genes. Correspondingly, 47% of single PC Hcs sequenced were members of the IGHV3 family. Likewise, 50% of IGKV germ-line genes belong to the IGKV1 family, and 50% of single PC  $\kappa$  sequences were also IGKV1.

Although the VH gene segments VH 3–23 and VH 4–34 are frequently over-represented, comprising up to 20% of the adult immune repertoire (53, 54), only 1/45 VH from patient 1 used either of these genes. However, there is evidence for preferential usage of specific IgV genes in our libraries, since multiple nonclonal VH used the same IgV gene. The best evidence for preferential usage is the SC library data in which no extrinsic selection bias is possible. For example, the germ-line gene IGHV1-18 is used by two unrelated single PCs, MCPC14 and 34, that use different D and J genes (Table 6) and also by three unrelated clones in the combinatorial library (Table 1). The repeated use of individual V genes by different B-cell progenitors is compatible with the positive selective power of a shared epitope of a common antigen.

**Somatic Mutation and Affinity Maturation of Response.** In a study of averaged values for 272 affinity-matured antibodies to both exogenous and self antigens, Andris and Capra (45) found average levels of mutation of IgG Hc V regions from germ line to be between 2 and 6%. All of the MC-derived sequences examined fell within this nominal range with the exception of  $\lambda$  Lcs from both patients 1 and 2,

which were only mutated 1% overall from germ line. This is consistent with a study of antigen-selected antibodies from ectopic follicles in rheumatoid synovia, which also expressed high-mutated VH and low-mutated VL  $\lambda$  (55). Mutations were more frequent within CDR regions as opposed to intervening framework regions for most sequences (Fig. 5), again as is canonical for affinity-matured antibodies (41–45). This correspondence between the mutation patterns of these patient tumor-derived Ig libraries and typical protein antigen-derived IgG antibodies again supports the inference of an affinity-matured host immune response against an intratumoral antigen. Furthermore, mutations in the nonclonal groups were similarly abundant and CDR-concentrated. This is compatible with the clones represented once in the sample (nonclonal) as being similarly antigen-selected and expanded but merely present at a lower average frequency in the tumor.

Central to this study is the hypothesis that the PC infiltrates in MC and infrequent NOS tumors are the result of a tumor antigen-specific response rather than a general inflammatory event, nonspecific proliferation, or recruitment of PCs through chemotaxis. Our index study of this question with two MC tumors showed repertoires of IgV genes in the tumors that were highly restricted and dominated by clonal groups. We observed abundant mutations that were clustered within the CDR regions compatible with an affinity-matured antigen-selected response typical of a protein antigen. Furthermore, IgG1 dominated the response where it was directly examined, reinforcing the inference of a protein origin for the neoantigen.

These results add evidence to the long-held inference that there is a tumor-derived antigen that elicits the characteristic B cell/PC immune response in MC and PC-infiltrated NOS tumors. Identification of the antigen (or antigens) may provide clues as to the etiology and/or biology of MC and, plausibly, of NOS tumors as well, in which the immune response may be less prominent because of ancillary features of the host immune environment. On the other hand, MC is intriguing in its histological resemblance to murine mammary tumors, which are caused by a retrovirus (56). A human mammary tumor virus, either exogenously transmitted or endogenously activated, could provide a unifying synthesis of data on etiology both of the tumor and of the host immune response. Whether this repertoire focus and the eliciting antigen extend to PC-infiltrated, NOS breast tumors are additional hypotheses that are the subject of our ongoing investigations. Finally, conclusions as to whether the neoantigen is etiologic in these breast tumors or is merely expressed as a consequence of the malignant transformation because of other causes will have to await its definitive identification.

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