Antigen-Driven Oligoclonal Expansion of Tumor-Infiltrating B Cells in Infiltrating Ductal Carcinoma of the Breast

Julia A. Coronella, Catherine Spier, Matthew Welch, Katrina T. Trevor, Alison T. Stopeck, Hugo Villar and Evan M. Hersh

*J Immunol* 2002; 169:1829-1836; doi: 10.4049/jimmunol.169.4.1829

http://www.jimmunol.org/content/169/4/1829

References

This article cites 42 articles, 11 of which you can access for free at: http://www.jimmunol.org/content/169/4/1829.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

Copyright © 2002 by The American Association of Immunologists All rights reserved.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Antigen-Driven Oligoclonal Expansion of Tumor-Infiltrating B Cells in Infiltrating Ductal Carcinoma of the Breast

Julia A. Coronella, Catherine Spier, Matthew Welch, Katrina T. Trevor, Alison T. Stopcek, Hugo Villar, and Evan M. Hersh

The objective of this study was to determine whether tumor-infiltrating B cells (TIL-B) of infiltrating ductal carcinoma (IDC) of the breast represent a tumor-specific humoral immune response. Immunohistochemical analysis of three Her-2/neu-negative IDC tumors from geriatric patients showed that TIL-B cluster in structures similar to germinal centers containing CD20+ B lymphocyte and CD3+ T lymphocyte zones with interdigitating CD21+ follicular dendritic cells, suggesting an in situ immune response. A total of 29, 31, and 58 IgG1 H chain clones was sequenced from the three IDC tumors, respectively. Intratumoral oligoclonal expansion of TIL-B was demonstrated by a preponderance (45–68%) of clonal B cells. In contrast, only 7% of tumor-draining lymph node and 0% of healthy donor PBL IgG H chains were clonal, consistent with the larger repertoires of node and peripheral populations. Patterns and levels of TIL-B IgG H chain somatic hypermutation suggested affinity maturation in intratumoral germinal centers. To examine the specificity of TIL-B Ig, a phage-displayed Fab library was generated from the TIL-B of one IDC tumor. Panning with an allogeneic breast cancer cell line enriched Fab binding to breast cancer cells, but not nonmalignant cell lines tested. However, panning with autologous tumor tissue lysate increased binding of Fab to both tumor tissue lysate and healthy breast tissue lysate. These data suggest an in situ Ag-driven oligoclonal B cell response to a variety of tumor- and breast-associated Ags. The Journal of Immunology, 2002, 169: 1829–1836.

Based on serum Ab reactivity with tumor cells and Ags, patient antitumor B cell reactions occur in >40% of breast cancer patients (reviewed in Refs. 10 and 11). A variety of breast tumor-associated Ags elicits naturally occurring serum Ab responses (reviewed in Ref. 10). Breast cancer-reactive B cells have also been identified in regional or draining lymph nodes of breast cancer patients (for examples, see Refs. 12–15). However, little direct evidence exists that TIL-B in breast cancer are tumor specific, although tumor cell-reactive TIL-B have been cloned from other tumor types, including melanoma, colon carcinoma, ovarian carcinoma, lung carcinoma, glioma, sarcoma, neuroblastoma, and Hodgkin’s lymphoma (16–23). One study found that anti-tumor Ag Abs were produced by TIL-B in ~70% of nonbreast tumors examined (19).

The best direct evidence for breast tumor cell-reactive TIL-B comes from a 1994 study by Katano et al. (24), in which a B cell line established from a human breast adenocarcinoma was shown to produce tumor cell-reactive Abs and inhibit growth of autologous tumor cells. However, human TIL-B reactivity is not limited to tumor-specific Ags; a study by our group found TNF-α-reactive Abs were produced by breast cancer TIL-B (20). Furthermore, a recent study demonstrated that Abs produced by TIL-B of typical medullary carcinoma (TMC) of the breast specifically bind β-acatin, which occurs on the surface of apoptotic TMC cells in vivo (25). It has also been demonstrated that TMC TIL plasma cells are the product of intratumoral oligoclonal proliferation and differentiation (8, 26). However, it is not clear that TMC is representative of other more common histologic types of breast cancer, as TMC has many unusual features, including a diagnostic plasmacytic infiltration, favorable prognosis, and expression of HLA-DR (27, 28).

Materials and Methods

Patient samples

Tumor tissue was obtained from three untreated women diagnosed with primary infiltrating ductal carcinoma (IDC). The three tumors obtained were Her-2 negative by immunohistochemistry. Tumor-draining lymph node from one of the three patients and peripheral blood from a healthy female donor were also acquired. Patient data are summarized in Table I. PBL were...
Table I.  Patient data and features of Ig G region construction

<table>
<thead>
<tr>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 3 Node</th>
<th>PBL Healthy Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>78</td>
<td>77</td>
<td>83</td>
<td>83</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>2.5</td>
<td>1.0</td>
<td>1.3</td>
<td>Node negative for tumor metastesas</td>
</tr>
<tr>
<td>Tumor grade</td>
<td>2</td>
<td>3</td>
<td>99/65</td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor/progesterone receptor % positive</td>
<td>50/0</td>
<td>0/90</td>
<td>1/0001</td>
<td></td>
</tr>
<tr>
<td>Axillary node status</td>
<td>1/15 positive</td>
<td>0/15 positive</td>
<td>0/25 positive</td>
<td>7 × 10^3</td>
</tr>
<tr>
<td>No. of lymphocytes used in RNA extraction for H chain library</td>
<td>1.4 × 10^4</td>
<td>3 × 10^5</td>
<td>4 × 10^5</td>
<td>7 × 10^5</td>
</tr>
<tr>
<td>Phage display Fab library (from separate RNA pool)?</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Phagocytosis of breast cancer cells by T cells and dendritic cells was studied. T cells from peripheral blood were isolated and cultured in the presence of breast cancer cells. The phagocytic activity of T cells was evaluated by flow cytometry using CD16 and CD56 as markers for natural killer cells. The results showed that T cells from patients with breast cancer had a significantly higher phagocytic activity than healthy donors. The phagocytic activity of T cells correlated with the stage of breast cancer, with higher activity observed in patients with advanced disease. These findings suggest that T cells play a role in the immune response to breast cancer. Further studies are needed to understand the mechanisms underlying this phagocytic activity and its potential as a therapeutic target.

DNA sequencing and analysis

Clones were sequenced using standard sequencing primers (Arizona Research Laboratories, University of Arizona) and resolved using the FAK-TORY program (Arizona Research Laboratories, University of Arizona). Candidate Ig G germline genes were identified via CHAINPOLYLOT (36). Percentage sequence identity was calculated by aligning the germline gene sequences to the cloned Ig G sequences. The DNA sequence and predicted protein sequence were submitted to GenBank (accession numbers: 1830 INFILTRATING B CELLS REACTIVE WITH BREAST CARCINOMA)

Constitution and panning of phage display library

Proteins for phage display library construction, panning, and analysis were obtained as previously described (34). Briefly, a 150-mg sample of tumor from patient 1 was homogenized with mortar and pestle in liquid nitrogen, and RNA extracted using TRIzol reagent (Sigma-Aldrich). RNA was further purified by lithium chloride extraction, and a total of 92 µg purified RNA was obtained. Of this, 20 µg RNA was used in a reverse-transcriptase reaction using the Superscript II First Strand system (Invitrogen). cDNA was treated with RNaseH (Invitrogen) and used directly in PCR amplification of H and L chain V regions, unrestricted for isotype. A Fab (H chain V region plus C H 1 and L chain V, plus C L 1) library of 1 × 10^6 Fab clones was cloned in the pCOMBx phage display vector (gift of C. Barbas, The Scripps Research Institute, La Jolla, CA). The phage-displayed library was panned with the MCF-7 breast cancer cell line in six sequential rounds of panning. In brief, ~4 × 10^12 phage were added to ~4 × 10^9 allogeneic breast cancer cells (MCF-7), incubated for 30 min at room temperature, centrifuged, and washed five times with PBS to remove unbound phage. Cell-bound phage was recovered by trypsin digestion (Invitrogen) and used to reinfert Escherichia coli strain XLI-B (New England Biolabs, Cambridge, MA). After overnight growth, phage was isolated by polyethylene glycol precipitation, and the cycle was repeated.

The phage library was also used with autologous tumor tissue lysate (soluble protein), with negative selection on healthy breast tissue lysate. Lysates were prepared by homogenization of snap-frozen tissue in liquid nitrogen, repeated freeze-thaw cycles, and extraction overnight at 4°C in PBS containing 1% Nonidet P-40 (Sigma-Aldrich), 10 µg/ml aprotinin (Sigma-Aldrich), and 1 mM PMSF (Sigma-Aldrich). ELISA plates (Costar, Garden Grove, CA) were coated overnight at 4°C with ~20 µg protein/well. Four wells were coated with tumor tissue lysate, and four wells with healthy breast tissue lysate. Plates were then blocked for 1 h with 5% BSA, and ~1 × 10^4 phage were added to each tumor tissue lysate well. Plates were incubated at 37°C for 2 h. Wells were washed with PBS + 0.1% Tween 20 (Sigma-Aldrich) to remove unbound phage, with increasing numbers of washes in successive rounds of panning. Phage were then eluted by the addition of glycine elution buffer (0.1 M glycine-HCl, pH 2.2), and neutralized with 3 M Tris. To reduce nonspecific binding, eluted phage were then added to healthy breast tissue lysate-coated wells for 30

Germ line nomenclature is as previously described (36).

Patient 3 TIL-B. One library was made with an IgG1-specific C region primer (CG102), and the second library was constructed using a pan-IgG C region primer (C H 1 IgG), as previously described (35). PCR products were cloned as described for patient 1.

After transformation of pGEM-T libraries into XL1-Blue competent cells (Invitrogen, Carlsbad, CA), random colonies were selected and grown overnight, and plasmid DNA was prepared with Wizard plasmid miniprep columns (Promega). Randomly picked clones were screened for the presence of IgG H chain insert through SacI/SacII (Promega) restriction digestion.

DNA sequencing and analysis

Clones were sequenced using standard sequencing primers (Arizona Research Laboratories, University of Arizona) and resolved using the FAK-TORY program (Arizona Research Laboratories, University of Arizona). Candidate Ig G germline genes were identified via CHAINPOLYLOT (36). Percentage sequence identity was calculated by aligning the germline gene sequences to the cloned Ig G sequences. The DNA sequence and predicted protein sequence were submitted to GenBank (accession numbers: 1830 INFILTRATING B CELLS REACTIVE WITH BREAST CARCINOMA)

Constitution and panning of phage display library

Proteins for phage display library construction, panning, and analysis were obtained as previously described (34). Briefly, a 150-mg sample of tumor from patient 1 was homogenized with mortar and pestle in liquid nitrogen, and RNA extracted using TRIzol reagent (Sigma-Aldrich). RNA was further purified by lithium chloride extraction, and a total of 92 µg purified RNA was obtained. Of this, 20 µg RNA was used in a reverse-transcriptase reaction using the Superscript II First Strand system (Invitrogen). cDNA was treated with RNaseH (Invitrogen) and used directly in PCR amplification of H and L chain V regions, unrestricted for isotype. A Fab (H chain V region plus C H 1 and L chain V, plus C L 1) library of 1 × 10^6 Fab clones was cloned in the pCOMBx phage display vector (gift of C. Barbas, The Scripps Research Institute, La Jolla, CA). The phage-displayed library was panned with the MCF-7 breast cancer cell line in six sequential rounds of panning. In brief, ~4 × 10^12 phage were added to ~4 × 10^9 allogeneic breast cancer cells (MCF-7), incubated for 30 min at room temperature, centrifuged, and washed five times with PBS to remove unbound phage. Cell-bound phage was recovered by trypsin digestion (Invitrogen) and used to reinfert Escherichia coli strain XLI-B (New England Biolabs, Cambridge, MA). After overnight growth, phage was isolated by polyethylene glycol precipitation, and the cycle was repeated.

The phage library was also used with autologous tumor tissue lysate (soluble protein), with negative selection on healthy breast tissue lysate. Lysates were prepared by homogenization of snap-frozen tissue in liquid nitrogen, repeated freeze-thaw cycles, and extraction overnight at 4°C in PBS containing 1% Nonidet P-40 (Sigma-Aldrich), 10 µg/ml aprotinin (Sigma-Aldrich), and 1 mM PMSF (Sigma-Aldrich). ELISA plates (Costar, Garden Grove, CA) were coated overnight at 4°C with ~20 µg protein/well. Four wells were coated with tumor tissue lysate, and four wells with healthy breast tissue lysate. Plates were then blocked for 1 h with 5% BSA, and ~1 × 10^4 phage were added to each tumor tissue lysate well. Plates were incubated at 37°C for 2 h. Wells were washed with PBS + 0.1% Tween 20 (Sigma-Aldrich) to remove unbound phage, with increasing numbers of washes in successive rounds of panning. Phage were then eluted by the addition of glycine elution buffer (0.1 M glycine-HCl, pH 2.2), and neutralized with 3 M Tris. To reduce nonspecific binding, eluted phage were then added to healthy breast tissue lysate-coated wells for 30
min, and unbound phage was recovered in panning rounds 3, 4, and 5. Phage was rescued by transfection of E. coli strain XLB-1 (Invitrogen). After overnight growth, phage was isolated by polyethylene glycol precipitation, and the cycle was repeated.

### Analysis of phage-displayed Ab pools by flow cytometry

Following selection of the phage Fab library on MCF-7 cells, phage pools were assessed by flow cytometry, as described (34). Cell lines MCF-7, 3133, 3199, and foreskin fibroblasts were analyzed. Cells were incubated with preselection phage display library, postselection phage display library, or anti-tetanus toxoid phage-displayed Fab as a negative control (tetanus toxoid, provided by C. Barbas, The Scripps Research Institute) (38). Fab reactivity was determined by flow cytometry using anti-M13 mouse mAb (Pharmacia) as secondary Ab, and FITC-labeled goat anti-mouse Fab’-specific Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) as tertiary Ab, as described (34). Ten thousand cells in the gated (live) population were counted per tube.

### Analysis of phage-displayed Ab pools by ELISA

Following selection of the phage Fab library on autologous tumor tissue lysate (soluble protein), phage pools were used in ELISAs against autologous tumor tissue lysate and healthy breast tissue lysate, as described (34). Plates were coated overnight with an excess of either autologous tumor tissue lysate or healthy breast tissue lysate (>20 μg) in 25 μl at 4°C, and blocked with 5% BSA in PBS. Phage (50 μl) that had been selected on autologous tumor tissue lysate were added to each well, incubated 2 h at 37°C, and washed 10 times with H2O, and HRP-conjugated anti-M13 Ab (Pharmacia) was added. Following a 1-h incubation at 37°C, plates were washed 10 times with H2O, and Fab binding was detected with ABTS substrate (Roche Molecular Biochemicals, Indianapolis, IN).

### Results

#### Histology and immunohistochemistry

The three cases of IDC of the breast were reviewed for TIL using H&E staining and immunohistochemical markers of B lymphocytes, T lymphocytes, plasma cells, and follicular dendritic cells. The tumors of patients 1 and 2 were largely composed of fibrous stroma, with interspersed islands of malignant cells (Fig. 1, A and D). The tumor of patient 3 consisted of large tumor nests interconnected by bands of fibrous stromal tissue (Fig. 1G). Although TIL were found scattered throughout the stroma and interspersed between tumor cells in all

![FIGURE 1. Histology and immunohistochemistry of IDC TIL. Serial sections of breast tumors from patients 1, 2, and 3 were stained for B cells (CD20), T cells (CD3), follicular dendritic cells (CD21), and cellular proliferation (Ki-67). A–I and K, ×200; J, ×400; and L, ×100. E, K, and L, Stained for both CD20 (red), cell surface; and Ki-67 (brown), nucleus.](http://www.jimmunol.org/)
Table II. Summary of molecular characteristics of IgG H chain repertoires

<table>
<thead>
<tr>
<th></th>
<th>No. of Sequences</th>
<th>No. with Stops</th>
<th>% with Stops</th>
<th>No. with 3-bp indel</th>
<th>% Seq Clonal</th>
<th>Groups With Diversity</th>
<th>Groups Without Diversity</th>
<th>% Mutation/R:S</th>
<th>FR1</th>
<th>CDR1</th>
<th>FR2</th>
<th>CDR2</th>
<th>FR3</th>
<th>Nonrandom R:S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>29</td>
<td>3</td>
<td>10.34</td>
<td>0</td>
<td>44.83</td>
<td>4</td>
<td>0</td>
<td>1.92/2.5</td>
<td>8.67/13</td>
<td>2.5/2</td>
<td>10.3/2.47</td>
<td>6.41/1.45</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Patient 2</td>
<td>31</td>
<td>7</td>
<td>22.58</td>
<td>1- to 3-bp deletion</td>
<td>67.74</td>
<td>3</td>
<td>0</td>
<td>4.08/0.0</td>
<td>11.93/2.56</td>
<td>4.49/2.22</td>
<td>13.71/1.47</td>
<td>6.81/0.83</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>Patient 3</td>
<td>58</td>
<td>7</td>
<td>12.07</td>
<td>1- to 3-bp insertion</td>
<td>62.07</td>
<td>8</td>
<td>3</td>
<td>2.01/1.03</td>
<td>10.97/2.28</td>
<td>3.28/1.08</td>
<td>9.45/2.31</td>
<td>6.38/0.28</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>Patient 3 node</td>
<td>26</td>
<td>4</td>
<td>15.38</td>
<td>0</td>
<td>7.69</td>
<td>0</td>
<td>1</td>
<td>2.35/2.5</td>
<td>8.41/1.67</td>
<td>2.56/1.44</td>
<td>8.59/3.56</td>
<td>4.89/0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy donor</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>0.0</td>
<td>0.32/0.40</td>
<td>0.32/0.70</td>
<td>0.36/2.0</td>
<td>0.29/3.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBMC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Number of individual clones sequenced from library.
- Number of sequences containing non-sense mutations.
- Number of sequences with in-frame 3-bp insertions or deletions.
- Percentage of the total number of sequences that belonged to clonal groups.
- Number of clonal groups that contained sequences with unique somatic mutations, i.e., intraclonal diversity.
- Number of clonal groups that contained only identical sequences, i.e., no intraclonal diversity.
- Percentage of mutation of IgV region from germline and ratio of replacement to silent mutation.
- Percentage of sequences with nonrandom numbers of silent and replacement mutations in FR and/or CDR, as calculated by the algorithm of Lossos et al. (42).
from patient 3, 62% belonged to one of the 11 identified clonal groups (Fig. 2).

The presence of unique and sequential somatic hypermutations in members’ clonal groups, examples of which are shown in Fig. 3, demonstrates definitively that B cell proliferation rather than PCR artifact produced the clonal groups. Somatic hypermutations were also far in excess of the calculated 0.2 PCR-induced mutations per V gene-encoded region. However, a small number of clones were identical, with no unique somatic mutations in comparison with each other, as might result from one or more cell divisions during which no somatic hypermutations were acquired. Most identical clones were verified as derived from identical B cell clones rather than PCR artifact, having been cloned from independent TIL pools. For example, the identical clones 1–18 and 2–38 were derived from libraries 1 and 2, respectively (Fig. 3A).

It was not possible to draw a genealogical tree for the fourth clonal group from patient 1 (Fig. 2), derived from a progenitor B cell using the germline V gene 4-39*06, because mutations were not linear. This may be explained by a phenomenon described by McHeyzer-Williams et al. (41), in which chimeric molecules produced during PCR confuse the linearity of descent for clones.

One IgG H chain library for patient 3 was produced with an IgG1-specific primer, and the second library with a pan-IgG-specific primer. All sequences were of the IgG1 isotype regardless of primer used, suggesting that the IgG1 isotype dominates the IgG TIL-B pool.

Only two reiterated sequences of 26 occurred in the tumor-draining lymph node sequences, for a total of 7.7% clonality (Fig. 2). No overlap existed between the sequences isolated from patient 3 TIL and patient 3 tumor-draining lymph node to suggest node/tumor trafficking (with the caveat that a larger sample size might be required to detect intersection). No repeated clones were observed in PBL sequences, consistent with expected low levels (<1/20,000) of reiterated clones for healthy donor peripheral blood (29) (Fig. 2).

**Germline V<sub>H</sub> gene usage**

V<sub>H</sub> germline gene family usage by TIL-B was analyzed for evidence of biased usage that might suggest epitope selection (Fig. 4). Library repertoires generated by this and similar methods have generally matched those generated by single cell RT-PCR (31, 8). The most common V<sub>H</sub>1 gene segments were used by TIL-B belonged to the V<sub>H</sub>3 and V<sub>H</sub>4 families. In addition, V<sub>H</sub>1 gene segments were used by 10–35% of TIL-B IgG and tumor-draining node IgG H chains, while no V<sub>H</sub>1 occurred in the PBMC repertoire.

![Clonal Group and Nonclonal](Image)

**FIGURE 2.** Clonality of TIL-B. Clonality of TIL-B was determined by IgG H chain sequence. PBMC, healthy donor PBMC; P3 Node, patient 3 tumor-draining lymph node; P3, patient 3 TIL-B; P2, patient 2 TIL-B; P1, patient 1 TIL-B. Clonal groups are indicated by open boxes, while sequences for which no clonal relatives were identified are indicated by shaded boxes. Numbers within boxes indicate the number of sequences in specific clonal groups or nonclonal group.

**Somatic mutation of TIL-B IgG H chains in germinal center reactions**

TIL-B IgG H chain V gene sequences were compared with progenitor germline V genes to determine levels and patterns of somatic hypermutation. Somatic mutations clustered in the CDRs, consistent with affinity maturation (Fig. 5). Mutation levels and patterns were similar to the tumor-draining lymph node-derived sequences and known affinity-matured Ab sequences (reviewed in Ref. 30), suggesting a functional ectopic germinal center reaction in the breast tumors. TIL-B IgG H chain CDR1 and CDR2 regions were mutated on average between 8 and 13%, respectively, in comparison with germline. Clones 1–18 shared a single common mutation from germline. B and C, Patient 2, clonal groups 3, 5, D and E, Patient 3 clonal groups 4, 8.
some Abs was nonrandom, as calculated by the method of Lossos et al. (42), suggesting affinity maturation (Table II and Fig. 6).

**Tumor binding by phage-displayed Ig derived from TIL**

To determine whether TIL-B Abs were reactive with tumor cells, a phage-displayed Fab library was generated from patient 1 TIL by the methods of Barbas et al. (34). The Fab library was panned with the allogeneic breast cancer cell line MCF-7. Binding of phage-displayed Fab to MCF-7 cells was measured by flow cytometry. Mean fluorescent intensity increased 2.7-fold from 13 to 36 after only four rounds of panning with MCF-7 cells, and 24-fold to a mean fluorescent intensity of 312 after six rounds of panning (Fig. 7). The MCF-7-selected Ab pool bound the 3199 breast cancer cell line with 8-fold greater intensity in comparison with the unselected Ab pool, and the 3133 breast cancer cell line with 2-fold greater intensity. In contrast, binding of TIL-B Abs to human primary fibroblasts decreased \( \sim 50\% \) after panning with MCF-7.

Phage-displayed TIL-B Fabs were also panned with autologous tumor tissue lysate (soluble protein). An \( \sim 7\)-fold increase in binding to autologous lysate was observed, as measured by ELISA against autologous tumor tissue lysate protein (Fig. 8). An equal enrichment for binding to healthy breast tissue lysate was also observed in the tumor tissue lysate-selected Abs.

**Discussion**

We examined the histology, IgG repertoire, and Ab specificity of TIL-B from three Her-2/neu-negative IDC of the breast derived from geriatric women. We found that in some cases, TIL-B aggregates resembled ectopic germinal centers containing CD21-positive follicular dendritic cells that were surrounded by a T cell zone (Fig. 1). However, unlike germinal centers from true lymphoid tissue, germinal centers contained no dark zone of Ki-67-positive proliferating B cells (Fig. 1). Mantle zones and CD38\(^{+}\) plasma cells were also absent.

Despite low Ki-67 staining of TIL-B, intratumoral oligoclonal expansion of TIL-B was established by the presence of clonal
groups derived from common progenitor B cells in the three breast tumors examined, regardless of histologic TIL-B density. The seemingly contradictory molecular and histologic proliferation data may reflect a low B lymphocyte proliferative rate over a period of time, or alternatively may be a relic of a previous period of proliferative activity and subsequent quiescence. Between 44 and 68% of IgG H chain sequences from TIL-B belonged to clonal groups, while only 7% of tumor-draining lymph node sequences and 0% of PBL sequences were clonal, consistent with the large primary human foreskin fibroblasts.

TIL-B IgH chain mutation levels, patterns, and germline gene usage suggest that TIL-B undergo affinity maturation intratumorally, presenting the possibility of production of high-affinity anti-tumor Ag Abs. However, this conclusion stems from indirect evidence of affinity maturation, which can only be resolved through Ag affinity studies. TIL-B IgH chains contained somatic mutations that clustered in the Ag-contacting CDRs, as is observed in affinity-matured Abs (reviewed in Refs. 30 and 43), and as was also seen in tumor-draining lymph node, but not peripheral blood IgG (Fig. 5). As calculated by the polynomial algorithm of Lossos et al. (42), replacement and silent mutations occurred nonrandomly in some TIL-derived Ig. A modest bias in usage of individual germline genes was consistent with epitope selection, with use of germline genes 1–18, 3–30, 4–39, and 4–61 by all three TIL-B repertoire (Fig. 4). Of these genes, only 3–30 is normally overrepresented in the peripheral repertoire of young or elderly adults (31, 44).

TIL-B-derived Fabs were reactive with the allogeneic breast cancer cell line MCF-7, indicating that TIL-B proliferate in response to tumor Ag rather than nonspecific inflammatory or cytokine signals (Fig. 7). Although Fabs were selected for binding to MCF-7, binding of these Abs to other breast cancer cell lines demonstrated the presence of reactive epitope(s) common to these cell lines. Although the TIL-B Fab pool selected against MCF-7 cell surface bound breast cancer cell lines preferentially in comparison with nonmalignant primary fibroblasts, the determination of true specificity awaits further characterization. Panning of the Fab library on autologous tumor tissue lysate (soluble protein) yielded Fabs with equal reactivity for soluble lysates from tumor and healthy breast tissue (Fig. 8). This indicates that at least some TIL-B produce Abs reactive with Ags shared by breast tumor and healthy breast.

Our study suggests that while TIL-B undergo tumor Ag-driven expansion in intratumoral follicles, deletion of autoreactive B cells may be deficient. Because few proteins expressed by tumor cells are truly tumor specific, the majority of Abs produced will likely be directed against autoantigens, and only a small percentage against tumor-specific Ags. Isolation of tumor-specific Abs from TIL will thus require judicious technique. We speculate that the lack of negative selection in intratumoral germinal centers may be the source of autoreactive breast tumor-associated serum Abs described by other groups, some of which are associated with pathologic autoimmune states (45, 46). Future investigations are needed to investigate the identities of Abs reactive with TIL-B Abs and implications of ectopic germinal centers in breast cancer.

References


22. Imahayashi, S., Y. Ichiyoshi, I. Yoshino, R. Eifuku, M. Takenoyama, and


15. Petrarca, C., B. Casalino, S. von Mensdorff-Pouilly, A. Rughetti, H. Rahimi,


15. Petrarca, C., B. Casalino, S. von Mensdorff-Pouilly, A. Rughetti, H. Rahimi,


22. Imahayashi, S., Y. Ichiyoshi, I. Yoshino, R. Eifuku, M. Takenoyama, and


15. Petrarca, C., B. Casalino, S. von Mensdorff-Pouilly, A. Rughetti, H. Rahimi,


22. Imahayashi, S., Y. Ichiyoshi, I. Yoshino, R. Eifuku, M. Takenoyama, and


15. Petrarca, C., B. Casalino, S. von Mensdorff-Pouilly, A. Rughetti, H. Rahimi,


22. Imahayashi, S., Y. Ichiyoshi, I. Yoshino, R. Eifuku, M. Takenoyama, and


15. Petrarca, C., B. Casalino, S. von Mensdorff-Pouilly, A. Rughetti, H. Rahimi,


22. Imahayashi, S., Y. Ichiyoshi, I. Yoshino, R. Eifuku, M. Takenoyama, and


15. Petrarca, C., B. Casalino, S. von Mensdorff-Pouilly, A. Rughetti, H. Rahimi,


22. Imahayashi, S., Y. Ichiyoshi, I. Yoshino, R. Eifuku, M. Takenoyama, and


15. Petrarca, C., B. Casalino, S. von Mensdorff-Pouilly, A. Rughetti, H. Rahimi,


22. Imahayashi, S., Y. Ichiyoshi, I. Yoshino, R. Eifuku, M. Takenoyama, and


15. Petrarca, C., B. Casalino, S. von Mensdorff-Pouilly, A. Rughetti, H. Rahimi,


22. Imahayashi, S., Y. Ichiyoshi, I. Yoshino, R. Eifuku, M. Takenoyama, and


15. Petrarca, C., B. Casalino, S. von Mensdorff-Pouilly, A. Rughetti, H. Rahimi,