Characterization of Lymphocytic Infiltrates in Normal, Preneoplastic, and Neoplastic Mouse Mammary Tissues

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ABSTRACT

Lymphocytic infiltrates were isolated from normal, preneoplastic, and neoplastic mammary tissues. The surface markers on the infiltrating lymphocytes were characterized by immunofluorescent staining and flow cytometry. Preneoplastic and neoplastic tissues contained 10- to 20-fold more in situ lymphocytes than did the normal pregnant gland. Most of these lymphocytes were T-cells. Relative to the T-cells in normal gland, the T-cells in C4 preneoplastic hyperplastic alveolar nodules and their spontaneous tumors have shifted in favor of the killer-suppressor subpopulation. This shift of T-cell subpopulations was a localized phenomenon and was not seen in the lymph nodes of hyperplastic alveolar nodules and tumor-bearing mice. C4 lesion infiltrating cells also contained a subpopulation of lymphocytes that expressed 5- to 6-fold more LFA-1 antigen (lymphocyte function associated antigen-1) than did normal lymph node cells. The infiltrating lymphocytes of mammary tumors from cloned cell lines, on the contrary, had the same staining profile as did the lymphocytes from normal gland. Since most studies with human breast cancer infiltrates have demonstrated increased killer-suppressor T-cells and the presence of activated lymphocytes (J. Hurlimann and P. Saraga, Int. J. Cancer, 35: 753-762, 1985; H. L. Whitwell, H. P. A. Hughes, M. Moore, and A. Ahmed, Br. J. Cancer, 49: 161-172, 1984; and J. A. Ledbetter, R. V. Rouse, H. Spedding Micklem, and L. Herzenberg, J. Exp. Med., 152: 280-295, 1980) the C4 hyperplastic alveolar nodules and spontaneous tumor system may be a more relevant model for studying breast cancer infiltrates.

INTRODUCTION

Mammary tumors of mice develop through distinct preneoplastic stages; thus they provide a system to study events associated with progression from preneoplastic to neoplastic tissue. Preneoplastic HANs were shown to be morphological precursors of mouse mammary cancers, and HAN lines (such as C4 and D2) were established which, when transplanted into mammary-gland free, syngeneic fat pads, gave rise to tumors at predicted frequencies (1). C4 and D2 HANs and their tumors share a common antigen(s) that is absent from normal mouse mammary tissue, as C4 and D2 preneoplastic hyperplastic mammary gland, the T-cells in C4 preneoplastic hyperplastic alveolar nodules and tumor-bearing mice. C4 lesion infiltrating cells also contained a subpopulation of lymphocytes that expressed 5- to 6-fold more LFA-1 antigen (lymphocyte function associated antigen-1) than did normal lymph node cells. The infiltrating lymphocytes of mammary tumors from cloned cell lines, on the contrary, had the same staining profile as did the lymphocytes from normal gland. Since most studies with human breast cancer infiltrates have demonstrated increased killer-suppressor T-cells and the presence of activated lymphocytes (J. Hurlimann and P. Saraga, Int. J. Cancer, 35: 753-762, 1985; H. L. Whitwell, H. P. A. Hughes, M. Moore, and A. Ahmed, Br. J. Cancer, 49: 161-172, 1984; and J. A. Ledbetter, R. V. Rouse, H. Spedding Micklem, and L. Herzenberg, J. Exp. Med., 152: 280-295, 1980) the C4 hyperplastic alveolar nodules and spontaneous tumor system may be a more relevant model for studying breast cancer infiltrates.

MATERIALS AND METHODS

Mice. All experiments used BALB/c mice inbred in our Animal Facility from breeding pairs originally obtained from the Cancer Research Laboratory, Berkeley, CA.

Preneoplastic Outgrowth Lines, Mammary Tumors, and Other Tissues. HAN line C4 was developed by Medina from a lesion originating in a 12-month-old, mammary tumor-bearing, female BALB/c mouse that had received 1.5 mg of dimethylbenz(a)anthracene between 8 and 10 weeks of age and had carried a pituitary isograft for 12 weeks (1). The BALB/c mouse is maintained by serially implanting small samples of HAN tissue into mammary fatpads that are cleared of host mammary tissue (4). The transplanted tissue grows and fills the fatpad with hyperplastic mammary tissue within 10-12 weeks. Tumors arise in 80% of C4 HAN implants within 26 weeks. Spontaneous C4 tumors are passaged no more than two times by s.c. implantation of tumor tissue into syngeneic recipients of either sex.

C4 lines 68H, 410.4, 66, and 168 were derived from a single spontaneously arising strain BALB/cFC3H mammary tumor (5, 6). They are routinely maintained in monolayer culture in Waymouth medium containing 10% fetal calf serum, 2 mm l-glutamine, penicillin (100 units/ml), and streptomycin (100 @g/ml). The tumor incidence in BALB/c mice with s.c. injection of 105 cells is 5% in 8-20 weeks for MMT line 68H, 75% in 3-8 weeks for MMT 66, and 100% in 4 weeks for both MMT 168 and 410.4 (7). Spontaneous metastasis in animals whose s.c. tumors were surgically removed was seen in 100, 100, and 80% of mice with MMT 68H, 66, 168, and 410.4 (8). Most (90%) of the 68H tumor cells express MMTV antigens on the cell membrane (7), and this may account for the low tumor incidence in MMTV negative BALB/c mice. However, 68H tumor tissue implanted s.c. in normal BALB/c mice grow to 10 x 10 mm in >90% of the recipients within 3 weeks. 68H tumors used in this experiment are first and second in vivo passage tumors. MMT 66, 168, and 410.4 expressed little or no MMTV antigens. Primary tumors from s.c. injection of 66, 168, and 410.4 cells were used in this study. All tumors were removed when less than 15 x 15 mm in size without visible necrosis.

Pregnant mouse mammary glands were obtained from 14- to 19-day pregnant BALB/c females. All five pairs of mammary glands were used.

BALB/c myeloma P3X63-Ag8.653 was purchased from the American Type Culture Collection.

Disaggregation of Mammary Tissues. All tissue culture reagents were obtained from Grand Island Biological Co. (Grand Island, NY) unless otherwise specified. Mammary tissues were removed aseptically, cut into 1-2-mm pieces, and digested with an enzyme cocktail containing collagenase Type III (3 mg/ml) (Cooper Biomedical, Malvern, PA), and deoxyribonuclease Type I (8 @g/ml) (Sigma, St. Louis, MO) in HBSS with 40% calf serum, in an orbital shaker (rotating at 250 cycles/
LYMPHOCYTIC INFILTRATES IN MOUSE MAMMARY GLAND AND LESIONS

min) for 60 min at 37°C. After incubation the supernatants were removed and replaced with fresh enzyme mixtures. The procedure was repeated. Cells were filtered through 45-μm Nytex (Tetko Inc., Elmsford, NY), washed free of enzyme, and resuspended in HBSS with 1% NCS for elutriation.

Production and Characterization of Monoclonal Antibody AMT 102. Hybridomas were produced from secondary in vitro sensitized lymphocytes by a modified method of Reading (9). A tumor arising spontaneously in C4 HAN tissue was dissociated by digestion with collagenase and DNase. Tumor cells were cultured in monolayer in SDME containing 10% heat inactivated fetal calf serum, 1% NCTC 109 (M. A. Bioproducts, Walkersville, MD), bovine crystalline insulin (8 μg/ml) (Sigma), 1 mM oxaloacetic acid, 0.5 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM minimal medium essential nonessential amino acids, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cultured tumor cells reached confluency in 2 days and were irradiated at 4000 rads with a cesium irradiator. Lymphocytes from the spleen of a C4 HAN bearer were suspended in SDME containing 30% mixed thymocyte conditioned medium at 2-3 × 10^6 cells/ml and added to the irradiated tumor cells. Mixed thymocyte conditioned medium was produced by coculturing equal numbers of thymocytes from 2-4-week-old BALB/c and C57BL/6 mice for 48 h in SDME with 50 μM 2-thymidine (Sigma). The culture supernatant was kept at −80°C until use. Lymphocyte cultures were left undisturbed for 6 days, when more than one-half of the surviving lymphocytes had become blasts. These cells were fused with BALB/c myeloma P3X63-Ag8.653 at a lymphocyte:myeloma ratio of 2:1. The fused cells were cultured with a feeder layer of thymocytes from normal BALB/c and C57BL/6 mice for 48 h in SDME with 50% 2-mercaptoethanol (Sigma), 20 μM hypoxanthine (Sigma), and 3 μM thymidine (Sigma). The culture supernatant was kept at −80°C until use. Lymphocyte cultures were left undisturbed for 6 days, when more than one-half of the surviving lymphocytes had become blasts. These cells were fused with BALB/c myeloma P3X63-Ag8.653 at a lymphocyte:myeloma ratio of 2:1. The fused cells were cultured with a feeder layer of peritoneal exudate cells.

Primary screening of antibody production by enzyme linked immunosorbent assay was performed with a mouse MoAb isotyping kit (Hyclone, Logan, UT). Supernatant containing mouse antibody was further tested for binding activity to various target cells using enzyme linked immunosorbent assay against target monolayers. All hybridomas obtained produced IgM. One of the 24 antibody producing colonies, AMT 102, produced high titer antibody and was found to bind non-specifically to a spectrum of adherent target cells, including BALB/c normal fibroblasts, normal mammary gland cells, D2 HAN, C4 tumor, and other mouse mammary tumor cells. However, this antibody did not bind to lymphocytes in the immunofluorescent assay.

Centrifugal Elutriation. Elutriations were performed with a Beckman J2-21 centrifuge using a JE-6B elutriation head with a standard chamber. The rotor speed was held constant at 2225 rpm throughout the procedure. The cells from dissociated mammary tissues were loaded, and two 100-ml fractions were collected at buffer flow rates of 6.5 and 15 ml/min. The cells remaining in the chamber were allowed to pellet and collected after the rotor stopped. All fractions were pelleted and resuspended in HBSS with 1% NCS for counting. Cells in fraction 2 (F2, collected at 15 ml/min) were used for lymphocyte characterization.

Macrophage Removal. Macrophages recovered in elutriation fraction 2 were removed by adherence to tissue culture Petri dishes (American Scientific Products, McGraw Park, IL) at a concentration of 2 × 10^6 cells/100-mm dish in DME with 10% NCS, L-glutamine (0.3 mg/ml), penicillin (100 units/ml), and streptomycin (100 μg/ml). The dishes were incubated at 37°C for 45 min. Non-adherent cells were collected, pelleted, and resuspended in 10 ml of DME containing 2% NCS, L-glutamine (0.3 mg/ml), and sodium azide (1 mg/ml).

Lymphocyte Labeling with FITC. LNC from normal BALB/c mice were labeled with FITC according to the method of Butcher and Weisman (10). Saturated FITC (Sigma, St. Louis, MO) solution was prepared by stirring an excess of crystalline FITC in phosphate buffered saline (KCl, 0.2 g/L; KH₂PO₄, 0.2 g/L; NaCl 8 g/L; Na₂HPO₄, 7H₂O, 2.16 g/L; pH 6.9) at room temperature for 2 to 3 h. After removal of undissolved material, the solution was diluted to 12% (approximately 70 μg/ml) with DME containing 2% NCS. Lymphocytes at 2.5 to 5 × 10⁷/ml were stained for 20 min at 37°C in the dark. FITC labeled LNC (FITC-LNC) were washed by centrifugation through 5 ml of calf serum in a 15-ml centrifuge tube at 300 × g for 10 min.

Identification of Lymphocyte Subpopulations by Immunofluorescent Assay. The antibodies used for lymphocyte characterization included rat MoAb directed to Thy 1.2, Ly 1, and Ly 2 (Becton Dickinson, Mountain View, CA). Hybridoma GK 1.5 was a generous gift from Dr. Frank Fitch, University of Chicago, Chicago, IL. Culture supernatant from GK 1.5 contains rat MoAb directed to L3T4 antigen (11, 12). Hybridomas M7/14 and M1/70, which produce rat MoAb to lymphocyte functional antigen, LFA-1 (13, 14), and macrophage surface antigen, Mac-1 (15), were purchased from the American Type Culture Collection (Rockville, MD), and culture supernatant was used for staining. The bound rat MoAb was visualized with the FITC conjugated F(ab')² fraction of mouse anti-rat Ig (Jackson Immuno Research Lab, Avondale, PA). Rabbit ASGM1 was purchased from Wako Chemical (Osaka, Japan), and FITC conjugated F(ab')² fraction of goat anti-rabbit immunoglobulin (Jackson Immuno Research Lab) was the second layer antibody. Mouse B-cells were identified by direct staining with the FITC conjugated F(ab')² portion of goat anti-mouse immunoglobulin (Jackson Immuno Research Lab). All hybridomas were maintained in SDME. For antibody staining 3 × 10⁶ cells were suspended in 100 μl of antibodies or medium. After 45 min incubation at 4°C, the first layer antibodies were removed by 3 washes and replaced by 100 μl of FITC conjugated second layer antibody. After another 45 min incubation at 4°C, cells were washed and resuspended in DME containing propidium iodide (30 μg/ml) (Sigma), which stained dead cells only. The cells were analyzed by flow cytometry 15 min after resuspension.

FACS 440 Analysis. Flow cytometry was performed by a dual laser FACS 440 using the 488-nm line of the 5-W argon laser for excitation. FITC and propidium iodide staining were measured simultaneously. The main excitation beam was blocked before the photo-multiplier tubes with filters LP520G and LP520D. The optical filter used for FITC fluorescence was BP530, with a 40-nm band width, and the filters for propidium iodide fluorescence were BP620, with a 40-nm band width, LP610G and LP610D. The sample pressure was set at 300–400 cells/second through a 70-μm nozzle.

All experimental data were acquired on list mode for four correlated parameters: forward scatter, 90° scatter; FITC, and propidium iodide fluorescence using the Los Alamos Cell Analysis System. The data were stored in magnetic tapes and later analyzed with a Consort 40 data analysis system. Dead cells that were stained with propidium iodide were eliminated, and the percentage of FITC stained viable cells was calculated. Non-specific staining by second layer antibody alone was subtracted.

RESULTS

Isolation of Lymphocytes from Dissociated Mouse Mammary Tumors. The lymphocyte separation procedure was validated on mixtures of FITC-LNC and dissociated cells from line 168 tumors. Lymph node cells prepared from normal BALB/c mice were labeled with FITC. MMT line 168 tumors were dissociated with collagenase and DNase. Viable cell numbers were determined by trypan blue exclusion, and 3.5 × 10⁷ cells of each type were mixed in a tube. The mixture was subjected to centrifugal elutriation. Two 100-ml fractions were collected at buffer flow rates of 6.5 and 15 ml/min (fractions 1 and 2). The cells left in the chamber were collected as fraction 3. Cell yields were 1 × 10⁷, 1 × 10⁶, and 2.8 × 10⁵ for fractions 1 to 3 (F1–F3), representing a total recovery rate of 70%. Over 90% of F1 cells are FITC-LNC. Fraction 2 contained 55% FITC-LNC. F3 were all unstained cells. Therefore FITC-LNC were found in F1 and F2 only.

The same elutriation procedure was used to isolate lymphocytes from dissociated mammary tissue. The cell recovery after elutriation varied depending upon the tumor tested. The total recovery (F1–F3) from seven experiments with 168 tumors was 83 ± 12% (SE), and that of nine C4 tumor experiments was 51 ± 16%. Cell morphology from each fraction was examined routinely using cytopsin preparation and Wright staining. Tit-
Lymphocytic infiltrates in mouse mammary gland and lesions

Sue infiltrating lymphocytes were found primarily in F2; F1 contained mostly RBC and debris. Therefore F2 cells isolated from dissociated tissues were used for lymphocyte characterization.

Fraction 2 contained lymphocytes with low 90° scatter and other cells with higher 90° scatter that may include macrophages, tumor cells, fibroblasts, etc. (data not shown). The adherent cells in this preparation were removed by 1 h incubation in plastic Petri dishes. The non-adherent cells were harvested for further analysis. Some of the cells with high 90° scatter did not attach during the 1 h incubation. To determine the nature of these non-adherent cells, mouse monoclonal antibody AMT 102, which bound to mouse mammary tumor cells and normal fibroblasts but not to lymphocytes, was used to stain the non-adherent F2 cells from dissociated 168 tumors. Cells were stained with AMT 102 and the FITC conjugated F(ab')2 fraction of goat anti-mouse Ig. Fig. 1 is the two-dimensional distribution profile of these cells, with log FITC on the x-axis and 90° scatter on the y-axis. Three different cell populations can be identified. Population A includes positively stained cells with high 90° scatter, and it represents 25% of total cells in this sample. Cells in population B are B-lymphocytes with low 90° scatter and are stained with the second layer antibody only. Population C has non-B-lymphoid cells. Based on these results, F2 cells with high 90° scatter were considered non-lymphocytes and were excluded in the computer data analysis of lymphocyte surface antigens.

Characterization of Lymphocytic Infiltrates in Normal, Preneoplastic, and Neoplastic Mouse Mammary Tissues. Single cell suspensions were prepared from various mammary tissues. As controls, lymph node cells were treated simultaneously with the same enzyme cocktails to ensure no antigen loss due to enzymatic treatment. The lymphocytic infiltrates recovered from elutriation fraction 2 were characterized by immunofluorescent assay. T-lymphocytes and their subpopulations were identified by MoAb directed to Thy 1.2, Ly 1, Ly 2, and L3T4. Control samples were stained with normal rat immunoglobulin. B-cells were stained directly with the FITC conjugated F(ab')2 fragment of goat anti-mouse immunoglobulin. Natural killer cells were detected with rabbit anti-ASGM1. Control samples were treated with normal rabbit serum. The results were analyzed by FACS 440. The staining profiles of lymphocytic infiltrates from C4 tumors are shown in Fig. 2. Panels 1 and 5 represent the forward and 90° scatter profiles of all viable cells. Dead cells stained by propidium iodide are excluded. The vertical bar in panel 5 separates lymphocytes with low 90° scatter from non-lymphocytes with higher 90° scatter. Only cells with low 90° scatter were analyzed for lymphocyte surface marker expression. Distinct peaks of positively stained populations can be identified from the unstained cells in panels 2 to 4 and 6 to 8. The percentage of positively stained cells in each sample was determined by computer analysis. Thy 1.2 positive cells were considered T-cells. B-cells were those stained with goat anti-mouse immunoglobulin. Asialo-GM1 positive cells may be non-adherent macrophages or NK cells (16). Control samples stained with normal rat immunoglobulin or normal rabbit serum were generally negative, and any non-specific staining (not exceeding 5%) was subtracted from the test sample.

Table 1 is a summary of the lymphocyte yield in Fraction 2 from various mammary tissues, including normal mammary glands from mid- to late pregnant BALB/c mice, C4 HAN and spontaneous C4 tumors, mouse mammary tumor line 68H, and other mammary tumors, including those of lines 66, 168, and 410.4. Lymphocyte yields were determined by multiplying the percentage of Thy 1.2 positive, surface immunoglobulin positive, and ASGM1 positive cells by the viable cell number in the lymphocyte preparation for each g of wet tissue weight. As can be seen, both preneoplastic and neoplastic mammary lesions had more substantial lymphocytic infiltrates than did the pregnant glands. In all of the tissue infiltrates examined, T-cells were the major constituents. The T-cell yields from the mammary lesions ranged from 3.5 ± 2.7 to 8.9 ± 6.4 × 10⁶ cells per g.

The infiltrating T-cells were further characterized by their expression of L3T4 (Th) and Ly 2 (Tc/S) antigens (Table 2). The percentage of L3T4 and Ly 2 positive cells divided by that

![Fig. 1. Identification of cells isolated from MMT 168 by centrifugal elutriation. Cells recovered in F2 from dissociated MMT 168 were analyzed on FACS 440 after labeling with monoclonal antibody AMT 102 and FITC conjugated F(ab')2 fragment of goat anti-mouse immunoglobulin. The x-axis shows log FITC, and the y-axis shows 90° scatter of the cells. Cells in population A were AMT 102 positive with high 90° scatter. Cells in population B were stained with second layer antibody only. Population C has unstained cells.

![Fig. 2. Staining profiles of C4 spontaneous tumor infiltrates. Cells recovered in F2 from dissociated C4 spontaneous tumors were analyzed on FACS 440 after indirect immunofluorescent staining with rat monoclonal antibodies to thy 1.2 (panel 2), Ly 1 (panel 3), Ly 2 (panel 4), L3T4 (panel 6), and rabbit anti-ASGM1 antiserum (panel 8). Direct immunofluorescent staining to detect surface immunoglobulin expression was by FITC conjugated goat anti-mouse immunoglobulin (panel 7). Panels 1 and 5 show the forward and 90° scatter of F2 cells. The x-axis shows log FITC, and the y-axis shows relative cell numbers.

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Viable lymphocytes are cells that have low 90° scatter and exclude propidium iodide. Consistent frequency, with B/T ratios ranging from 0.2 to 0.3, were detected throughout all of the tissues examined at a significantly lower than that of normal pregnant gland (2.4 ± 0.7) infiltrates. The same shift in favor of α was not seen in the lymph node cells from C4 HAN or tumor bearing animals, but were enriched for Ly2 bearing cells. The B/T ratio was similar (0.2 to 0.3) in infiltrates from normal, preneoplastic, and neoplastic tissues. ASGM1 positive cells were found in all tissues examined. A portion of the infiltrating lymphocytes in C4 HAN and tumors was activated, as shown by enhanced LFA-1 expression.

DISCUSSION

Lymphocytic infiltrates in normal, preneoplastic, and neoplastic mouse mammary tissues were isolated by centrifugal elutriation and characterized by immunofluorescent staining. Preneoplastic and neoplastic tissues contained 10- to 20-fold more in situ lymphocytes than did the normal pregnant gland. Most of these lymphocytes were T-cells. Relative to the T-cells in normal gland, the T-cells in C4 HANs and their tumors were enriched for Ly2 bearing cells. The B/T ratio was similar (0.2 to 0.3) in infiltrates from normal, preneoplastic, and neoplastic tissues. ASGM1 positive cells were found in all tissues examined. A portion of the infiltrating lymphocytes in C4 HAN and tumors was activated, as shown by enhanced LFA-1 expression.

The validation experiments for the centrifugal elutriation procedure demonstrated recovery of FITC-LNC in both fraction 1 and fraction 2. The FITC-LNC in fraction 2 had slightly higher forward scatter than those in fraction 1 (data not shown). When cells dissociated from mammary tissues were separated by the same methods, lymphocytes were found primarily in fraction 2. These lymphocytes usually showed higher forward scatter than did collagenase- and DNase-treated, control, normal lymph node cells. One can postulate that HAN and tumor infiltrating lymphocytes are larger because they are activated. A number of reports have associated functional activities with mammary tumor infiltrating lymphocytes, including enhancement of tumor growth (17, 18), inhibition of tumor growth (19), and suppression of lymphocyte blastogenic responses (20). Since 32 and 52% of C4 HAN and C4 tumor infiltrating lymphocytes, respectively, show enhanced LFA-1 expression, these cells appear to be activated. However, one can not exclude other lymphocytes (14). Since most of the infiltrating lymphocytes in the mammary lesions were T-cells, it was of interest to determine the activation state of these cells. Fig. 3 illustrates the staining profiles of normal LNC and C4 HAN and C4 tumor infiltrating lymphocytes by anti-LFA-1. All LNC were stained, and the peak of cell distribution moved up about 20 channels after anti-LFA-1 staining (Fig. 3, panel I). There are two subpopulations of lymphocytes in C4 HAN and C4 tumor infiltrating lymphocytes, as defined by anti-LFA-1. The dimly stained subpopulation expressed amounts of LFA-1 similar to those of normal LNC. The fluorescent intensity of the second subpopulation was 5.1 (C4 HAN) and 6.4 (C4 tumor)-fold brighter, equivalent to the amount of LFA-1 expressed by cytolytic T-cells described by Kurzinger et al. (14). C4 tumor contained more highly LFA-1 positive cells (52%) than C4 HAN (32%). This experiment has been repeated in its entirety with similar results.

**Table 1** Lymphocyte content in normal, preneoplastic, and neoplastic mouse mammary lesions

<table>
<thead>
<tr>
<th>Source of infiltrates</th>
<th>T-cells</th>
<th>B-cells</th>
<th>ASGM</th>
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<tr>
<td></td>
<td>% of L3T4</td>
<td>% of Ly2</td>
<td>L3T4/Ly2/B/T</td>
</tr>
<tr>
<td>C4 HAN (5)</td>
<td>47 ± 15 a</td>
<td>44 ± 8</td>
<td>1.2 ± 0.6 b</td>
</tr>
<tr>
<td>C4 spontaneous tumor (3)</td>
<td>49 ± 12 a</td>
<td>47 ± 9</td>
<td>1.1 ± 0.4 c</td>
</tr>
<tr>
<td>68H tumor (3)</td>
<td>65 ± 12 a</td>
<td>51 ± 3</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>Other mammary tumors (6)</td>
<td>75 ± 8 a</td>
<td>52 ± 2</td>
<td>2.5 ± 0.8</td>
</tr>
<tr>
<td>Normal pregnant gland (2)</td>
<td>59 ± 26 a</td>
<td>64 ± 3</td>
<td>2.4 ± 0.7</td>
</tr>
<tr>
<td>Normal lymph nodes</td>
<td>71 ± 9 a</td>
<td>52 ± 3</td>
<td>2.8 ± 0.6</td>
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**Table 2** Characterization of lymphocytic infiltrates from normal, preneoplastic, and neoplastic mouse mammary lesions

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* Mean ± SE.
* a b P < 0.05 by Student's t-test, compared to normal pregnant gland.
* c P < 0.02 by Student's t-test, compared to normal pregnant gland.
other possible explanations; e.g., tumor cells may produce proteolytic enzymes which modify the membrane structure of the infiltrating lymphocytes, resulting in different forward scatter and elutriation properties.

Our finding that 10- to 20-fold more lymphocytes exist in mammary lesions than in normal glands is consistent with that of others (21, 22). As reported before from this laboratory (23, 24) and other laboratories using either experimental mammary tumors (20) or human breast cancer tissues (22, 25, 26), T-cells are the predominant infiltrating cell type. The relative number of TH and TC/S cells can be expressed as the ratio of L3T4/Ly2. This ratio was lower for C4 HAN and C4 tumor (1.2 ± 0.6 and 1.1 ± 0.4) than for normal pregnant mammary gland (2.4 ± 0.7). However, the L3T4/Ly2 ratios for line 68H tumors (2.1 ± 0.5) and mammary tumors of line 168, 410.4, and 66 tumors (2.5 ± 0.8) were similar to that of normal gland (2.4 ± 0.7). Our laboratory has attempted previously to determine the relative number of TH and TC/S cells in several mammary tumor lines by means of cytotoxicity assays with monoclonal antibodies to Ly1 and Ly2 purchased from New England Nuclear (Boston, MA). With that method, the Ly1/Ly2 ratio in 68H tumors was about 3, whereas it was less than 1 for other mammary tumors lines, including 168 and 410.4 (24). We have retested the anti-Ly1 and anti-Ly2 monoclonal antibodies from New England Nuclear and found the ratio of Ly1/Ly2 for normal BALB/c lymph node cells to be 3.3 by immunofluorescent staining and flow cytometry and 1.4 by cytotoxicity assay. Ledbetter et al. (27) have reported greater numbers of Ly2 positive cells and a smaller number of Ly1 positive cells determined by cytotoxicity assays than by immunofluorescent assays using normal mouse lymphocytes. It was speculated that some cells expressed low levels of Ly1, detectable by flow cytometry, but not sufficient to mediate lysis in the cytotoxicity assay. The cause of higher numbers of Ly2 positive cells in the cytotoxicity assay was not clear. In any event these two different lymphocyte assay systems may account for the discrepancy observed in our present studies versus those we reported earlier, although other technical differences may also be attributable. In our previous work lymphocytes had been isolated by isokinetic gradient separation, rather than by centrifugal elutriation.

Lymphocytes associated with human breast lesions have been studied in frozen tissue sections by immunohistological methods (22, 25, 26) and in suspension after tissue dispersion (21). The findings are generally consistent, with certain exceptions. As mentioned above, breast cancer tissues are associated with much heavier infiltrates than are their normal or benign counterparts. About 75% of the infiltrates, whether they are from normal, benign, or cancerous tissues, are T-cells. Some of the tissue infiltrating T-cells are activated since they expressed Ia antigen (25), HLA-DR antigen (22, 28), or IL-2 receptor (21, 29). The percentage of activated T-cells in the total T-cell populations, however, ranges from 14% (21) to 30% (25) in different studies. The TH/TC/S ratios of cancer tissue infiltrates, in contrast to peripheral blood leukocytes and benign tissue infiltrates, are usually in favor of the TC/S population (25). Nevertheless, there are always significant numbers of exceptions in which the TH/TC/S ratios are similar to those of peripheral blood leukocytes (25). The TH/TC/S ratios are not related to tissue histology or tumor grading (25). The level of NK cells is usually very low in breast cancer tissue (25, 30).

The variations in TH/TC/S ratios from individual human breast cancer may reflect tumor progression or different tumor properties yet undefined or may be attributed to trivial technical differences. These variations, however, make the selection of a relevant animal model system a difficult task. We have examined several mouse mammary tumor systems, including neoplastic lesions and their spontaneous tumors, as well as mouse mammary tumor lines. The neoplastic HAN contains an equivalent number of infiltrates as tumors (HAN tissue contains a large proportion of fat cells; therefore the lymphocyte yield per g of tissue is actually an underestimation relative to tumors) and may represent a model for early cancerous tissue to be distinguished from benign breast tissue. The high infiltrate contents, the presence of activated lymphocytes, and the TH/TC/S ratios in C4 HAN and tumors were similar to those of most human breast cancer tissue. The presence of NK cells may reflect the early stage of the mouse lesion in tumor progression. The functional analysis of NK cells from HAN and tumor infiltrates is the subject of another report.

T-cell infiltrates from tumors of established lines were similar to those from normal, pregnant mammary glands and to normal lymph nodes. Unlike C4 HANs and their tumors, these tumors grow to 10 x 10 mm from s.c. injection in 2—3 weeks; their infiltrates may reflect lymphocyte influx into rapidly proliferating mammary tissues, with little influence from the tumor associated antigens or other tumor related factors. Furthermore, the infiltrates from tumors of these cell lines were similar despite the very different biological behavior of these tumors (7). These results may indicate that these rapidly growing cell lines are insufficient models for the characterization and study of immune cells from most human breast tumors, although they may be model systems for those exceptional breast cancer cases mentioned above.

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LYMPHOCYTIC INFILTRATES IN MOUSE MAMMARY GLAND AND LESIONS


Characterization of Lymphocytic Infiltrates in Normal, Preneoplastic, and Neoplastic Mouse Mammary Tissues

Wei-Zen Wei, Kevin Malone, Keith Mahoney, et al.


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