Identification of T and B Lymphocytes in Human Breast Cancer With Immunohistochemical Techniques

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Cryostat sections of 50 breast cancer specimens and several lymphoid organs were investigated with antisera against human T-lymphocyte antigen, human lymphocytes, and human immunoglobulins using the immunofluorescence technique and the immunoperoxidase technique. These methods proved to make it possible to discriminate between T and B cells in lymphocytic infiltrates in tissue sections. In nearly all mammary carcinomas studied, T cells were found to predominate in and around tumor cell nests. Only the intraductal carcinomas of this series contained a substantial number of B cells in addition to T cells. The presence of T cells indicates that the host–tumor interaction *in vivo* may—at least for some of the tumors studied—be attributed to a cell-mediated immune reaction. The role of the B cells found in the lymphocytic infiltrates of the intraductal carcinomas is still a matter of speculation. Moreover, these findings enhance the value of established histologic classifications. These classifications may have to be modified to provide them with a more functional basis. (Am J Pathol 84:529–544, 1976)

LYMPHOCYTIC INFILTRATION is quite common in human female breast cancer and has been demonstrated to be a favorable prognostic sign.[1-8] This is analogous with other malignancies, e.g., seminoma and melanoma.[9] Evidence is accumulating that both humoral [10-12] and cellular [13-19] immune reactions are involved in the host–tumor relationship. There are strong indications that T lymphocytes play a key role in the *in vitro* destruction of neoplastic cells in breast cancer.[20-22] The possible role of B cells in the tumor–host relationship is less clear. Only a few reports have been published on the characterization of lymphocytes in breast cancer tissue specimens. These studies, however, were based on results obtained by either cell isolation followed by rosette techniques for the subdivision of lymphocytes or by tissue homogenization followed by estimation of the total immunoglobulin content of the homogenate as a measure of the presence of plasma cells.[23,24]

Because selective loss of immunocompetent cells cannot be ruled out with these methods and because these methods provide only indirect evidence of the number of T and B lymphocytes, we have tried to identify

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these cells directly in tissue sections using immunohistochemical techniques. This paper presents our results with these techniques using specific antisera against a) lymphocytes, b) human T-lymphocyte antigen (HTLA), and c) immunoglobulins in order to identify T and B cells.

Materials and Methods

Patients

Patients' material was randomly collected from several pathology departments where the frozen section procedure for rapid diagnosis is practiced. Fifty consecutive cases from which malignant tumor tissue became available in this way were used for this study. None of the patients received preoperative treatment. There was no evidence of detectable organ metastases in any of these cases. The age of the patients ranged from 27 to 81 years (mean age, 60.5 years), with only 6 patients below the age of 48 (being the menopausal breakpoint in the age-specific incidence curve).[25] No efforts were made to estimate the age of the tumors in any patient, as there is no reliable way to determine the tumor age.[26]

Preparation of Tissues

Breast Tissues

An approximately 1 × 1 × ½ cm piece of the tumor margin of each breast cancer specimen was snap-frozen in liquid nitrogen (−196 C), and the remaining tissue was fixed in 10% buffered formalin (pH 7.2). As much as possible, macroscopically, an area containing both tumor and some surrounding normal breast tissue was selected.

Subgross patterns of growth were not considered, as they lack a clear correlation with histologic features like lymphocytic infiltrate.[27] The tumor masses of all carcinomas studied ranged from 2 to 3.5 cm. For reference purposes, histologically normal tissue outside the lesions was obtained from 3 patients with benign breast disease (e.g., fibroadenomatous lesions).

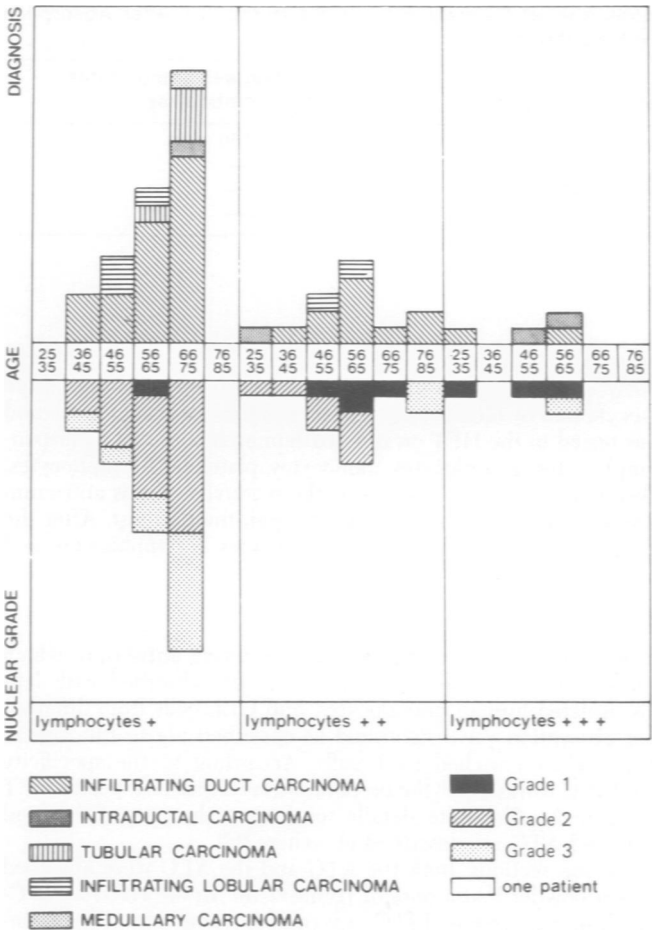
Lymphoid Tissues

From fresh autopsies (less than 4 hours post mortem) samples of normal thymus, spleen, and lymph node were used for specificity control as the topographic distribution of different types of lymphoid cells in these organs is quite well known.[28,29] None of the patients from whom these organs were removed were known to have had breast cancer or immunologic disorders. These tissues were handled in the same way as the breast cancer specimens.

Light Microscopy

The formalin-fixed breast tissue specimens were routinely dehydrated and embedded in paraffin. Sections 4 μ thick were stained with hematoxylin and eosin, periodic acid–Schiff, and methyl green–pyronine. When no formalin-fixed tissue was available, 4-μ cryostat sections were fixed in formadex [30] and were stained similarly. The sections were classified histologically. Cytonuclear grading was restricted to those criteria that seemed relevant to the aim of our study. The guidelines as suggested by Fisher *et al.*[31] were roughly followed for tumor typing.

We distinguished the five histomorphologic tumor types mentioned in Text-figure 1. Nuclear characteristics were expressed in three grades as follows: Grade 1, poorly differentiated pleomorphic nuclei of large size, with prominent nucleoli and frequent mitoses; Grade 2, intermediate nuclear characteristics; Grade 3, well-differentiated, small and



TEXT-FIGURE 1—Summary of results of histologic studies on human breast cancers in relation to age and lymphocyte infiltration.

uniform nuclei. Special attention was paid to the amount of lymphocytic infiltrate which was semi-quantitatively measured in the following way: 1, few lymphocytes, scattered in and around the tumor mass; 2, moderate number of lymphocytes; 3, many lymphocytes, sometimes in clusters situated in the stroma around the tumor.

Antisera

All tissue specimens were tested for the presence of immunoglobulins and cells bearing HTLA and human lymphocyte antigen. Both the direct immunofluorescence technique (IFT) and the indirect immunofluorescence technique (IIFT) were used. Fluorescein isothiocyanate (FITC)–conjugated rabbit antisera against human IgA, IgM, and IgG (Dakopatts, Denmark) were used in the IFT, and an antiserum against human IgD (CLB) was used in the IIFT. IgG fractions of rabbit antisera specific for peripheral blood lymphocytes [antilymphocyte globulin (ALG)] and thymus lymphocytes [anti-T lymphocyte globulin (ATG)] were used to demonstrate lymphocytes and T cells, respectively. In short, the ALG and ATG were prepared and their specificity was established as follows.

Table 1A—Titers of Antibodies Against Different Blood Cells in the IIFT After Absorption of Antilymphocyte Serum With Erythrocytes

Test cells	Before absorption	After absorption with granulocytes and thrombocytes
Lymphocytes	1280	160
Monocytes	640	—
Granulocytes	640	—
Thrombocytes	320	—

Antilymphocyte Globulin

Rabbits were immunized with isolated human lymphocytes. The resulting antiserum, which was not spontaneously specific for lymphocytes, was repeatedly absorbed with human red cells (made leukocyte free by filtration) [32] and isolated human granulocytes and platelets. The antiserum was tested in the IIFT on isolated human blood cells in suspension as well as in smears [lymphocytes, granulocytes, monocytes, platelets, T lymphocytes, and chronic lymphatic leukemia (CLL) cells]. Moreover, the reactivity of this antiserum with various plasma proteins was established with a passive agglutination test. After the above absorptions, the serum proved to react only with lymphocytes, T lymphocytes, and CLL cells (Table 1).

Anti-T lymphocyte Globulin

Rabbits were immunized with human T lymphocytes. The resulting antiserum, which was not spontaneously specific for T lymphocytes, was repeatedly absorbed with leukocyte-free human red cells, isolated human granulocytes, and CLL cells from different donors. The specificity after absorption was established as described above but also in lymphocyte suspensions depleted or enriched in T cells. According to the specificity criteria proposed by a WHO/IARC workshop,[33] the preparation is considered specific for T lymphocytes in the IIFT (Table 2). Elaborate details concerning the preparation and specificity testing of the ALG and ATG are described elsewhere.[34,35]

Before they were used on tissue sections, both the ATG and the ALG were absorbed with acetone-dried human liver powder [36] at a dose of 10 mg/1 ml. In the IIFT, a FITC-labeled swine antirabbit IgG (Dakopatts; SwaR-FITC) was used as second layer. Occasionally, a horseradish peroxidase (HRP)-labeled horse antirabbit immunoglobulin (HoaR-HRP) was applied as second layer. The IgG fraction of this antiserum was prepared according to the procedure described by Avrameas and Ternynck.[37] The HoaR-HRP was visualized by incubation with diaminobenzidine (DAB) and H_2O_2 according to Graham and Karnovsky,[38] followed by treatment with osmium. For double staining procedures, tetramethylrhodamine isothiocyanate (TRITC)-labeled horse antirabbit Ig (HoaR-TRITC) was used as a second or fourth layer. Working dilutions, batch numbers etc. are listed in Table 3.

Table 1B—Titers of Antibodies Against Plasma Proteins in the Passive Agglutination Test Using Human Erythrocytes Coated With Various Plasma Proteins After the Above Mentioned Absorptions

IgG	IgM	IgA	Albumin	Fibrinogen
4	—	—	—	—

Table 2A—Titers of Antibodies Against Different Blood Cells in the IIFT After Absorption of Anti-T-Cell Serum With Erythrocytes

Test cells	Before absorption	After absorption with granulocytes, CLL cells and IgG isolation of the anti-T-cell serum
Thymocytes	640	640
CLL cells	160	10
Granulocytes	160	10
Monocytes	160	10
Lymphocytes	320	640
Thrombocytes	80	—

CLL = chronic lymphatic leukemia.

Immunofluorescence Technique

Both IFT and IIFT procedures were preformed as previously described.[39] Tissue sections incubated with normal rabbit serum instead of a rabbit antiserum served as controls for the IIFT. In multilayer incubation, either of the following procedures was adopted: a) Incubation with ATG followed by SwaR-FITC and subsequently incubation with ALG followed by HoaR-TRITC. b) Incubation with ALG followed by HoaR-TRITC and subsequently with FITC-labeled anti-IgM.

To estimate the T and B cell composition of lymphocytic infiltrates, serial incubations on subsequent slides of the same area were made using different antisera. All incubation steps were alternated with 30-minute phosphate-buffered saline (PBS; pH 7.4) washings. It should be mentioned that the incubations for the direct IFT were performed for 60 minutes and those for the indirect IFT for 30 minutes. Slides were read within 24 hours on a Leitz Orthoplan fluorescence microscope with incident illumination (filter combination Nos. 3 and 4 of the Ploem Opak).[40,41] A mercury lamp (Philips CS 150W) was used for excitation. Fluorescence micrographs were made on Ansco 200 and Kodak Tri-X pan.

Results

Histologic Findings

Reference Tissues

Neither the lymphoid organs studied—i.e., the thymus, the spleen, and the lymph node—nor the nonpathologic breast tissues showed any histomorphologic abnormalities.

Classification of Tumors

Of the 50 tumors studied, 36 were classified as infiltrating duct carcinomas (Figure 1). Five tumors belonged to the group of the lobular

Table 2B—Titers of Antibodies Against Plasma Proteins in the Passive Agglutination Test Using Human Erythrocytes Coated With Various Plasma Proteins After the Above Mentioned Absorptions of the Anti-T-Cell Serum

IgG isolation of the anti-T-cell serum	IgG	IgM	IgA	IgD	Albumin	Fibrinogen	κ	λ
Before	128	32	256	64	32	512	—	—
After	—	—	—	—	—	—	—	

Table 3—Working Dilutions, Batch Numbers, etc., of Antisera Used

Antiserum	Code	Obtained from	Labeled with	Dilution in PBS
Rabbit antihuman IgG	F 1090 lot No. 075	Dakopatts	FITC	1:75
Rabbit antihuman IgM	F 1091 lot No. 035	Dakopatts	FITC	1:50
Rabbit antihuman IgA	F 1092 lot No. 015	Dakopatts	FITC	1:100
Rabbit antihuman IgD	KH 20-07-A01	CLB	—	1:30
Swine antirabbit IgG	F 2190 lot No. 1271	Dakopatts	FITC	1:120
Rabbit antihuman lymphocyte globulin		CLB	—	1:40
Rabbit antihuman thymocyte globulin		CLB	—	1:40
Horse antirabbit Ig	17-2-T_2	CLB	TRITC	1:40
Horse antirabbit IgG	17-2-H	CLB	HRP	1:160
Normal rabbit serum	KO-23	CLB	—	1:40

FITC = fluorescein isothiocyanate, TRITC = tetramethylrhodamine isothiocyanate, HRP = horseradish peroxidase, PBS = phosphate-buffered saline, CLB = Central Laboratory of the Netherlands Red Cross Blood Transfusion Service.

invasive carcinomas. There were four so-called tubular carcinomas and one medullary carcinoma. Finally, there were four intraductal carcinomas (Figure 2), which did not show any infiltrative growth in the available tissue blocks. No (pure) colloid carcinomas were present in this series.

Text-figure 1 summarizes the results of the histologic studies on these tumors in relation to age. Eight tumors fulfilled the criteria for nuclear Grade 1, 24 for Grade 2, and 18 for Grade 3. The degree of lymphocytic infiltration per tumor appeared rather variable, but a statistically significant ($0.01 < P < 0.025$) and positive correlation was found with the nuclear grade (i.e., Grade 1 tumors and many lymphocytes often occur together). Moreover, a trend was observed for Grade 1 tumors to occur more frequently in relatively young patients. Nine tumors showed a marked increase in the number of plasma cells in the infiltrates. This increase, however, was only slight compared with the increase in numbers of lymphocytes, and no relationship to a particular tumor type was found. Usually the plasma cells were lying at the border of the tumor, mainly in the perivascular areas. In the tissue specimens from 3 patients, normal or slightly atrophic lobules were found lying close to the tumor mass, their intralobular stroma crammed with lymphocytes. One intraductal carcinoma with very dense lymphocytic infiltrate contained a small follicle with a germinal center.

Fluorescence Patterns

Lymphatic Tissues

Cytoplasmic Fluorescence. In the lymph node and spleen, plasma cells with a clearly fluorescent cytoplasm were frequently found with

antisera against IgG, IgA, and IgM in all of the areas known to contain plasma cells. Occasionally an IgD-positive plasma cell was found. In the thymus only a few plasma cells were observed in the medullary region. They exclusively contained IgM and no IgA, IgG, or IgD.

Noncytoplasmic Fluorescence. A rim-like fluorescence pattern quite unlike the cytoplasmic fluorescence was observed on small lymphocytes with ALG and ATG. This was also the case with anti-IgM. Data obtained from fluorescence studies on peripheral lymphocytes have indicated that most B cells carry IgM as well as IgD on their membranes; some B cells, however, carry only IgD.[42-44] Our efforts to use anti-IgD as a B-cell marker in tissue sections were not successful because the contrast between the faint rim-like fluorescence of the small lymphocytes and the slight nonspecific fluorescence of the connective tissue was not enough.

Thymus. It was found that in both the thymic cortex and the medulla, virtually all small lymphocytes showed a rim-like fluorescence with both ATG and ALG. Since thymus lymphocytes lie closely packed together, a honeycomb pattern was obtained (Figure 3). The medulla of the thymus contained a small number of cells showing the same rim-like fluorescence with anti-IgM. No cells showing this pattern were observed in this area with anti-IgG or anti-IgA sera. The epithelial cells were strictly negative with all antisera used.

Lymph Node and Spleen. Many IgM-bearing cells with the above-mentioned rim-like fluorescence pattern could be observed. These cells were situated mainly in the marginal zones of the follicles (Figure 4) and to a lesser extent in the interfollicular areas. Rim-like fluorescence of lymphoid cells was very seldom observed with anti-IgG and anti-IgA in lymph nodes and spleen. Nearly all lymphocytes located in the periarteriolar lymphocyte sheath of the spleen and the paracortical areas of the lymph node (Figure 5) were shown to be HTLA positive. Finally, it could be demonstrated that the presence of HTLA-positive cells was not restricted to the areas mentioned. Occasionally, they were found in the outer cortical zones of the lymph node or around (and sometimes even in) the germinal centers (Figure 6) of both spleen and lymph node. Since our findings with ATG and anti-IgM are in agreement with the established concepts on the distribution of T cells and B cells in lymphatic tissues, we refer to lymphoid cells positive with anti-HTLA as *T cells* and to lymphoid cells positive with anti-IgM as *B cells* as far as the rim-like fluorescence pattern is concerned. Both T and B cells showed a rim-like fluorescence with the ALG; plasma cells, however, were negative with both ALG and ATG. With the multilayer incubation technique, it was not possible to identify all lymphocytes belonging either to the T or B cell sub-

populations. As was to be expected from studies on peripheral blood lymphocytes, a few ALG-positive cells were negative with both anti-HTLA and anti-IgM sera. Moreover, some cells were found to be positive for both HTLA and IgM, confirming the known facts about cells with mixed T and B cell characteristics.

Nonpathologic Breast Tissue. The three normal breast tissue specimens studied contained varying amounts of lymphocytes. The variation may be attributed to changes related to menstrual cycle and age. These lymphocytes were always identified as T cells. They were only found in the loose stroma around lobules and ducts. Sometimes T cells were observed between epithelial cells of both ductules and acini (Figure 7). No B lymphocytes were detected, and only few plasma cells were found. The plasma cells were positive for IgA or IgM in about equal numbers. No IgG- or IgD-containing plasma cells were observed.

Breast Cancer Tissue

Cytoplasmic Fluorescence. Incubation with the anti-Ig sera mentioned revealed the presence of a small but relatively constant number of plasma cells containing IgA and IgM (Figure 8) in most of the tumors. Occasionally an IgG-positive plasma cell was found.

Noncytoplasmic Fluorescence. ATG cells with a rim-like fluorescence pattern were found in all tumors. Sometimes they occurred sparsely in the interstitial tissue. Frequently they were found lying in rows between collagen bundles. It was observed that these T cells sometimes showed marked pleomorphism, their form varying from rounded to elongated (spindle cell–like) and sometimes having rather angular outer contours (Figure 9). Again, T-cell clusters gave the impression of a honeycomb (Figure 10). All lymphocytes situated in tumor cell nests were identified as T cells with this technique (Figure 11). Of the 50 tissues studied, only the four intraductal carcinomas showed a substantial amount of B cells in the lymphocytic infiltrates (Figure 12). The dense lymphocytic infiltrate in the lobules close to the tumor border also appeared to consist of T cells.

Discussion

The results of this study indicate that investigation of frozen tissue sections with the immunofluorescence technique used with specific antisera against immunoglobulins, lymphocytes, and T lymphocytes enables us to establish the distribution of T and B cells in tissue specimens. The use of specific heteroantisera in the immunofluorescence technique to identify T and B cells has been described by others.[45–49] The extensive testing of these antisera on lymphoid organs reported in this paper,

however, has contributed towards a more reliable interpretation of the observations in tissue sections with unknown distribution of T and B lymphocytes. We were able to trace the lymphocytes with ALG, and by changing filter combinations we could subsequently identify them as T cells with ATG when the multilayer procedure was used. The same procedure was followed for the B cells, using ALG and aIgM, respectively. The theoretical possibility of cross reaction occurring between the different antisera could not be excluded. Apparently these reactions did not interfere, since only seldom could a slight overlap of staining be demonstrated on lymphocytes in subsequent slides of lymph nodes, when ATG and aIgM were used, respectively. This overlap may be mainly attributed to the mixed membrane properties of some lymphocytes. All breast cancer specimens investigated with the immunohistochemical techniques were shown to contain lymphocytes in about the same variation as seen light microscopically (Table 1), except that more lymphocytes were observed due to the greater sensitivity of the detection method. The interepithelially situated lymphocytes or T cells were especially easy to detect, as they could not be mistaken for, e.g., pyknotic tumor cell nuclei in the IFT, whereas this mistake is inevitable in light microscopy. Moreover, it could be demonstrated in all infiltrative carcinomas that virtually all lymphocytes positive with ALG were also positive with ATG, thus confirming their T-cell nature. The number of B cells was negligible in this group of tumors. From this study it may be concluded that, assuming the phenomena observed are representative of immunologic events caused by tumor–host interactions, mammary carcinomas trigger the immune system to react in a certain amount of cases with a predominantly T-cell–mediated response. Any changes in time of tumor cell antigenicity and/or immune reactivity of the patient are still a matter of speculation, as the data obtained from this study represent only a certain moment in a process. If we consider the differences in speed of growth as an important biologic feature of mammary carcinomas, it can be assumed from our observations that the more pleomorphic and probably more rapidly growing tumors have something to do with a pronounced local T-cell response in the relatively young patients, as these phenomena appeared to occur together quite frequently. Part of these facts could be explained by the usually more active cellular responsiveness in younger persons [50–52] and the stronger antigenicity of the more anaplastic and rapidly growing tumors as postulated by some workers.[53] These observations stress the importance of studying the tumor cells as well as the environment (host) in which they develop. The presence of T cells in normal mammary gland tissue may be attributed to a possible surveillance role, in which antigenic

information from the glandular lumen and/or from altered cell surfaces is developed. This hypothesis would fit the observations of Marsh [54] in large-bowel mucosa of mice. Regarding the facts obtained from *in vitro* studies,[20] the T cells in and around the carcinomas will possibly have a specific cytotoxic function. At this moment, our findings do not have direct clinical implications. We hope to achieve this with an expanded investigation comparing the T and B cell distribution in mammary carcinomas, the regional lymph nodes, and peripheral blood of breast cancer patients.

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[*Illustrations follow*]

Legends for Figures

Figure 1—Infiltrating mammary carcinoma showing close contact between lymphocytes and pleomorphic tumor cells. (H&E, × 225).

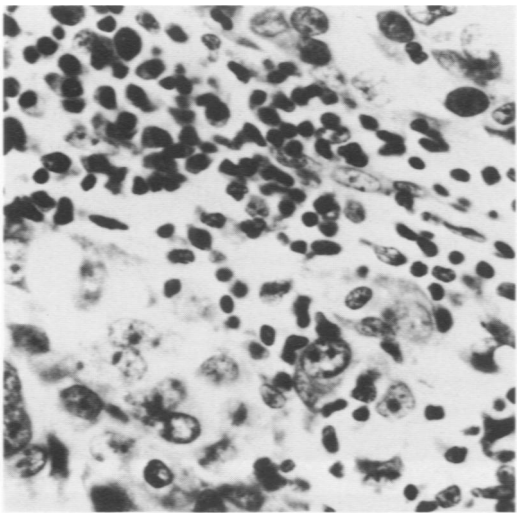
Figure 2—Intraductal carcinoma surrounded by a dense lymphocytic infiltrate (H&E, × 225).

Figure 3—Human thymus; cortical zones with densely packed lymphocytes positive with anti-HTLA serum (ATG and SwaR-FITC, × 225).

Figure 4—Human lymph node incubated with aIgM showing a follicle. Coarse deposits of IgM are seen in the germinal center. Rim-like fluorescence of B lymphocytes in the follicle border zone. (aIgM-FITC, × 90)

Figure 5—Paracortical area of a human lymph node incubated with ATG. Scattered around a small vessel (postcapillary venule), many positive lymphocytes are seen. (ATG and SwaR-FITC, × 225)

Figure 6—In (*arrow*) and around the germinal center of a lymph node follicle, T lymphocytes are found (ATG and HoaR-HRP, × 112).



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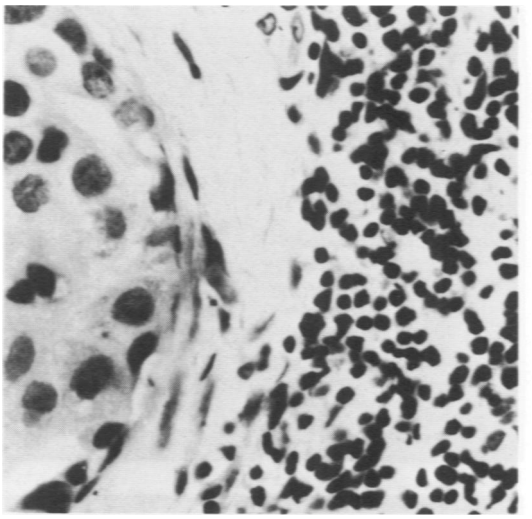

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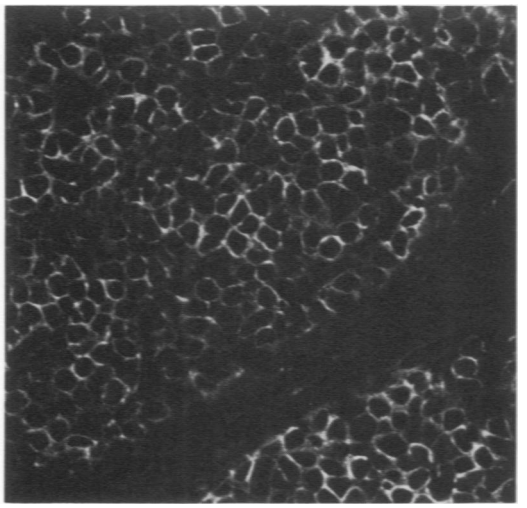

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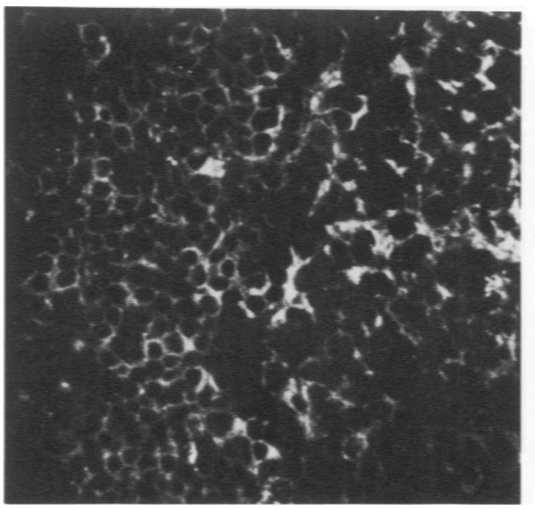

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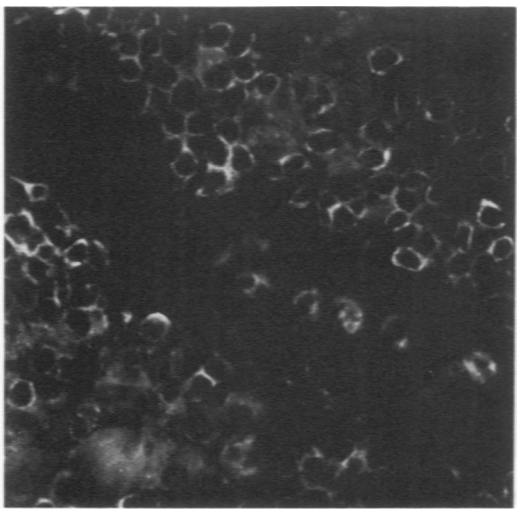

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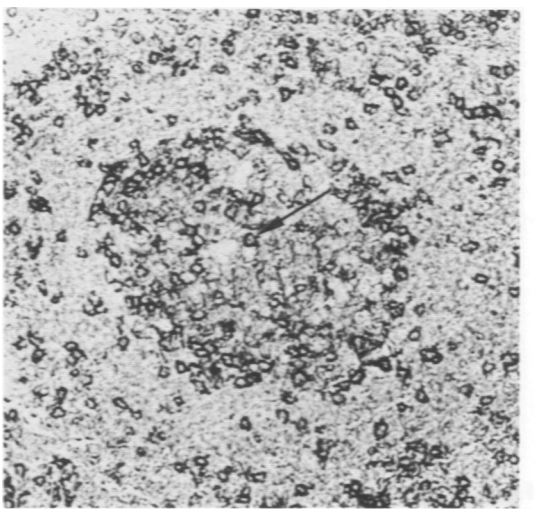

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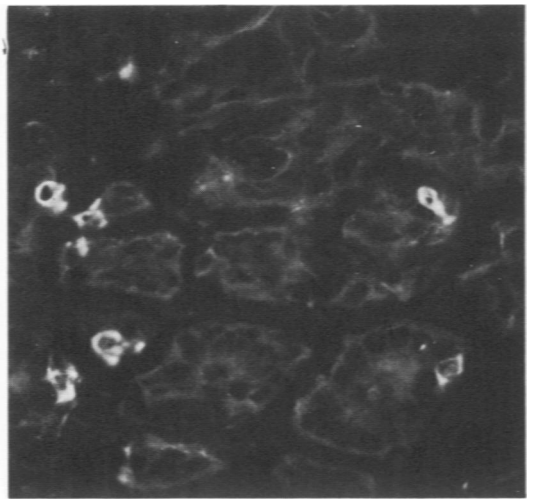

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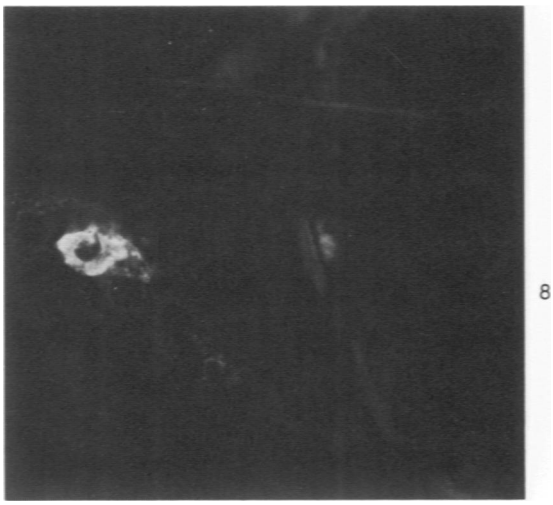

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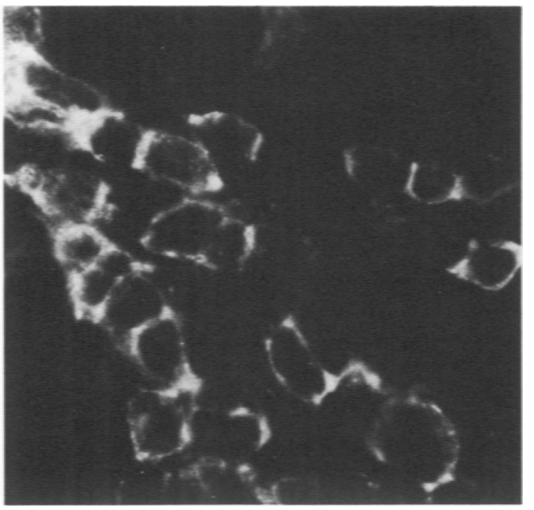

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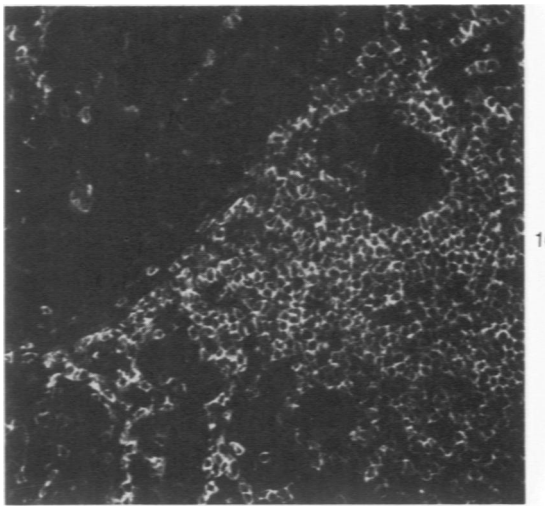

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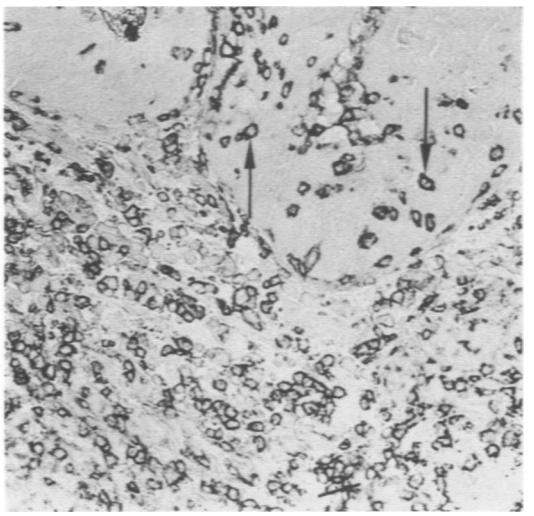

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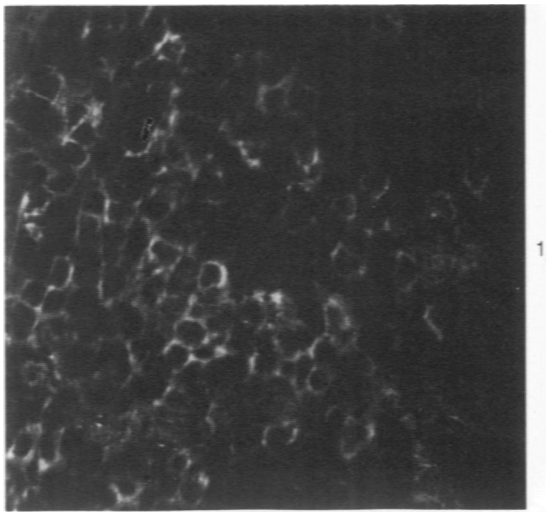

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Figure 7—Normal human breast lobule showing the interepithelial localization of T lymphocytes (ATG and SwaR-FITC, × 225). **Figure 8**—IgM-bearing plasma cells, showing cytoplasmic fluorescence, in the interstitial tissue of breast cancer (aIgM-FITC, × 225). **Figure 9**—T lymphocytes at the tumor border of an infiltrative mammary carcinoma showing pleomorphic contours (ATG and SwaR-FITC, × 900). **Figure 10**—Dense T-lymphocyte infiltrate around cancer cell nests (ATG and SwaR-FITC, × 90). **Figure 11**—T lymphocytes visualized with the immunoperoxidase technique. *Arrows* indicate the localization between tumor cells. (ATG and HoaR-HRP, × 112) **Figure 12**—Anti-IgM-positive B lymphocytes scattered in a lymphocytic infiltrate around an intraductal carcinoma (aIgM-FITC, × 486).