Reactive Changes in Breast Carcinoma and in Axillary Lymph Nodes

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The cell infiltration in breast carcinoma (stromal reaction) and the reactive changes in the axillary lymph nodes were investigated and assessed together with the production of factor inhibiting Ehrlich ascites-cell migration and suppressor factor. We used methods of morphology, immunohistochemistry and migration inhibition assay were applied. The study comprised 144 women with breast carcinoma. Their age at operation varied from 32 to 70 years with the mean (standard deviation) of 50.5 (9.9) years. Among 742 axillary lymph nodes from 144 patients, 125 axillary lymph nodes from 32 patients did not react and we examined the remaining 617 axillary lymph nodes from 112 patients. Reactive changes were found in 282 (45.7%) of these lymph nodes, most of which were in T-cell region (paracortical type) and T-cell and B-cell region (mixed type). The active paracortex in the lymph nodes was related with production both of a factor inhibiting Ehrlich-cell migration and a suppressor factor. We have shown that inhibitory factor was produced from erythrocyte rosette-forming cells and the subset T+ (non-rosetting T-cells after incubation of theophylline) was stimulated in vitro with breast-cancer extract. The erythrocyte rosette-forming cells were separated from peripheral blood mononuclear cells and lymph node lymphocytes. The cells found in the subset T+ (non-rosetting T-cells) produced suppressor factor. The latter could inhibit the proliferation and differentiation of CD8 T-cytotoxic lymphocytes, and possibly damage their function.

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Introduction

Abundant lymphocytic infiltration in breast carcinoma has been shown as a predictor linked with favorable prognosis for the patients.1 Its unfavorable significance with respect to the tumor growth, recurrence rate, and the involvement of lymph nodes has been considered as well.2 T-cells are the major component of the tumor-infiltrating lymphocytes (TILs) of the breast cancer, and the predominance of CD8 T-cells in the mononuclear cell infiltrates was considered as an indicator for a more favorable prognostic sign.3 It has been shown that TILs were functionally suppressed in vivo, and a few of them (<10%) expressed receptors for IL-2.4 The majority of TILs showed a reduced response to PHA.5 Freshly isolated TILs revealed an impaired cytotoxic response, and did not have detectable cytotoxicity to autologous tumor cells.6 The cytotoxic function of TILs or TALs (tumor-associated leukocytes) may be blocked by inhibitory signals locally produced by tumor cells.7 Moreover, a few mononuclear cells expressing mRNA for cytokines IL-2, IL-2R, TNF-α, IFN-γ were observed in the stroma of breast carcinomas.8

Syrjänen9 showed a correlation between paracortical activity in the regional lymph nodes and the 5-year survival rate of the patients. Unfavorable sign was "lymphocyte depletion"—hypocellular lymph nodes with fibrosis or hyalinosis.10 The paracortical activity was defined on the basis of the extent of the paracortex, density of lymphocytes and the height of the post-capillary venule (PCV) endothelium.11 The prognostic significance of the germinal centers in breast carcinoma was less informative than the extended deep cortical region (paracortical area).12

The purpose of this study was to investigate and assess the cell infiltration in the breast carcinoma and the types of reactive changes in regional (axillary) lymph nodes together with production of factor inhibiting the migration of Ehrlich-ascites cells, as target tumor cells, and suppressor factor from peripheral blood and lymph node erythrocyte rosette-forming cells (ERFCs), stimulated with breast-cancer extract. The data obtained could contribute to better explain the paracortical activity in the lymph nodes as determine by the generation and proliferation of lymphocytes of different T-cell subsets, including suppressor cells influencing the release of suppressor factor.

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Material and Methods

Patients

The subjects were 144 breast cancer patients treated between 1986 and 2003 at surgical clinics of Pleven University School of Medicine and Sofia Medical University. All of them underwent curative modified radical mastectomy and axillary node dissection, and their age at operation varied from 32 to 70 years with the mean (standard deviation) of 50.5 (9.9) years. The removed breast carcinomas referred to pT1-2N0-1Mx according to TNM system. A written informed consent was obtained from each patient. The lymph nodes of the levels of I-III were dissected through their small axis in two equal halves and were used for morphological and immunological examination.

Histological examination

Tissue slices from tumor and lymph nodes were fixed in 10% neutral formalin (pH 7.0) for 24 hr and embedded in paraffin. Deparaffinized sections were stained with hematoxylin and eosin (H&E), v. Gieson, methylgreen pyronin, using PAS method (Hotchkiss).

The reactive changes in axillary lymph nodes were evaluated by using our system for assessment. According to this system, T- and B-cell regions of lymph nodes were graded in four degrees (0-III) depending on their size, cell activity and cell transformation. The dimension of the paracortex, i.e. the length between the outer cortex and the medulla, was determined as follows: in Grade 0, up to 400 μm (without reactive changes); in Grade I, from 401 to 700 μm; in Grade II, from 701 to 1000 μm; and in Grade III, 1001 μm or more. The active paracortex is characterized by densely populated small lymphocytes and numerous post-capsillary venules with high cuboidal endothelial cells. The germinal centers of lymph node follicles as the most reactive structures of B-cell regions were measured by their transversal dimensions: in Grade 0, up to 200 μm; in Grade I, from 201 to 300 μm; in Grade II, from 301 to 500 μm; and in Grade III, 501 μm or more. Centroblasts and macrophages were found in reacted germinal centers. Sinus histiocytosis was graded from 0 to III degree in accordance with the amount of macrophages in the lymphatic sinuses. The frequency of plasmacytes in the medullary cords (plasmacytosis) was graded as plasmacytes 0 (0-10%) of plasmacytes, Grade I (21-50%), Grade II (51-80%) and Grade III (over 80%).

The reactive changes in axillary lymph nodes were classified into the following 3 types: (1) isolated reactive change in T-cell regions (paracortical, T type), (2) reactive change in B-cell regions (B type) and (3) reactive change in T- and B-cell regions (mixed, TB type).

Immunohistochemical techniques

Immunohistochemical examination of tumor sections was made with monoclonal and polyclonal antibodies. The monoclonal antibodies used for cryostat sections were: anti-CD3 (anti-Leu-4) Becton Dickinson, OKT4 (anti-CD4) and OKT8 (anti-CD8) Ortho-mune, and anti-CD19 (anti-Leu-12) Becton Dickinson. The following polyclonal antibodies were used for paraffin sections: anti-CD3 rabbit anti-human-PAP (kit Dako), anti-lgG, IgA, IgM, anti-kappa and anti-lambda light chains, anti-lysozyme, and anti-ε1-anti-trypsin (Biogenex Laboratory)-rabbit anti-human PAP.

Cell preparations

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood of patients with breast cancer and patients with fibrocytic disease as controls. The centrifugation at 800 x g was performed for 15 min over a cushion of lymphocyte separation medium (Polysep, Pharmachim Bulgaria). The PBMCs partially depleted adherent cells by incubation in plastic flasks at 37°C for 35 min. The lymph node suspensions were prepared in RPMI 1640 from minced lymph nodes which were passed through a nylon meshwork to filter and remove adherent cells. The ERFCs from PBMCs and lymph node cells were separated by rosetting with sheep erythrocytes, and were freed of attached erythrocytes by treatment with Tris-buffered 0.83% ammonium chloride (pH 7.2). Suspension of ERFCs was prepared in RPMI 1640 supplemented with 10% heat-inactivated pooled human serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cytocentrifugationgraphic analysis of ERFCs obtained from PBMCs was performed by using a cytocentrifuges FACStar (Becton Dickinson). The frequency of T cells (CD2, CD3, CD4, CD8) and NK cells (CD3, CD57 and CD3, CD16, CD56) was determined.

A procedure for separating T-cell population according to the lability of their E receptor to theophylline was used. ERFCs were incubated with theophylline (10^-4 M) for 60 min at 37°C. After washing twice rosetting with sheep erythrocytes was performed again and the subsets Tα and Tγ were separated. Tα-rosetting, theophylline-resistant lymphocytes functioned as helper/inducer cells for pokeweed mitogen B-cell to differentiate into plasma cells. Tα-nonrosetting theophylline-sensitive lymphocytes, which failed to rosette, functioned as suppressors for this differentiation. The frequency of CD4 and CD8 T-cells in the two subsets was determined using monoclonal antibodies anti-CD4 and anti-CD8. In the normal control persons, Tα contained 71.8 ± 4.7% of OKT4 (CD4) and 16.5 ± 3.8% of OKT8 (CD8), while Tγ contained 23.1 ± 4.5% of OKT4 (CD4) and 53.0 ± 2.9% of OKT8 (CD8).

Preparation of cancer extract and stimulation of lymphocytes

Cancer extract was obtained by using an extraction procedure with hypertonic potassium chloride as was described previously. Briefer, ten milliliters of cold 3 M KCl in PBS (pH 7.2) was added to every 1 g of finely minced tumor (breast cancer) tissue for 16 hr. The mixture was centrifuged at 40,000 x g for 1 hr at 4°C. Further centrifugation of the mixture at 18,000 x g was also performed. The supernatants were dialyzed against PBS, and the protein content was adjusted to 0.35-0.50 mg protein/mL. Extracts of benign breast tissue from patients with fibrocytic disease of the breast were also prepared.

Samples of 0.4 mL suspension of ERFCs, and Tα and Tγ-cells,
each containing $8 \times 10^7$ cells ($2 \times 10^9$/mL), were mixed with 80 $\mu$L of cancer extract or extract of benign breast tissue and were incubated for 24 hr at 37°C in a 5% CO$_2$ atmosphere. The mixtures were centrifuged at 18,000 $\times$ g for 20 min at 4°C. The supernatants were decanted and then prepared for examination of migration inhibition activity as was reported previously. $^{21}$ ERFCs from 20 cancer patients were stimulated with extract of benign breast tissue, while those from 12 control patients with fibrocystic disease of the breast were stimulated with cancer extract.

**Assessment of migration inhibition**

Ehrlich ascites tumor maintained in BALB/c mice by every 10-day serial i.p. transplantation ($1 \times 10^7$ cells) was the source of target cells. Samples of 0.2 mL of Ehrlich ascites cells ($2 \times 10^9$/mL) suspended in RPMI 1640 supplemented with 5% fetal calf serum and antibiotics were mixed with 0.1 mL of the supernatants of ERFCs, T$_i$ and T$_s$ subsets. The mixtures were incubated for 2 hr at 37°C in the atmosphere of 5% CO$_2$. Capillary tubes sealed one end were filled with Ehrlich cells and were centrifuged at 250 $\times$ g. The portions with sedimented cells were cut off, placed in chambers filled with medium and incubated at 37°C for 24 hr. The areas of Ehrlich-cell migration were projected and measured with planimeter. We defined the migration index (Mgi) as the ratio of two average areas:

$$Mgi = \frac{\text{Average areas of migration of Ehrlich cells incubated with supernatant}}{\text{Average areas of migration of Ehrlich cells incubated without supernatant}}$$

Mgi less than 0.80 was considered as significant. On average 10 capillary tubes were used to examine each supernatant. Inactive supernatant, which did not inhibit the migration of Ehrlich cells, was mixed with supernatant showing inhibitory effects (0.1 mL + 0.1 mL). The effect of the combination on the Ehrlich-cell migration was examined.

**Statistical analysis**

Frequency of reactive changes by region, and the association of type of reactive changes and type of cell infiltration were analyzed by chi-square test, while the migration index was compared among groups by Mann-Whitney U test, and SPSS (for Windows, release 10.07) was used for the calculations.

**Results**

**Cell infiltration in tumor (stromal reaction)**

The cell infiltration in the breast carcinomas, examined in 112 cases of invasive breast cancer with reactive changes in axillary lymph nodes, was classified into four groups; three groups were defined by the type of infiltrating cells while the remaining one group was defined by scanty or missing of cell infiltration.

The polymorphocellular infiltration was found in 13 cases of invasive ductal carcinoma and in one atypical medullary carcinoma. $^{27}$ The infiltrate consisted of lymphocytes, plasmocytes, polymorphonuclear neutrophil and eosinophil leucocytes, macrophages and mastocytes, and was diffuse or focal.

Lymphocytes mixed with a few plasmocytes were predominant in the cell infiltrate of the cancers in 15 invasive ductal carcinoma, 3 medullary and 3 atypical medullary carcinoma. The infiltrate was diffuse, but focal cell infiltration was also observed around cancer nests, tubules and small vessels frequently with prominent endothelial cells. In the atypical medullary carcinomas the infiltrate was disposed marginally and between tumor sheets. Lymphocytes within small tumor nests were sometimes detected.

Plasmocytes, which were mostly found around tumor sheets, predominated in the stromal infiltrate in 10 invasive ductal carcinomas.

The cell infiltration of tumors was scanty or missing in 67 invasive carcinomas: 57 ductal, 8 lobular, 1 secretory and 1 cribriform. The sparse infiltrate contained lymphocytes, plasmocytes and sometimes macrophages. The stroma of the tumor had an appearance of a dense fibrous connective tissue with hyalinisation around tumor nests and tubules.

In addition, in 24 (21.4%) patients who underwent chemotherapy primarily, areas of necrosis, stromal fibrosis and limited cell infiltration were observed in the carcinomas. The changes in tumor cells were: enlarged nuclei showing pleomorphism, hyperchromasia, prominent nucleoli, necrotic change, giant cell formation and vacuoles in the cytoplasm.

**Immunohistochemistry of the cell infiltrate in the tumors**

In carcinomas with predominant lymphocytes, about 80-90% of positive lymphocytes were revealed in the cell infiltrate by using monoclonal antibody anti-CD3 on cryostat sections. Marked CD3$^+$ T-lymphocytes in the atypical medullary carcinomas were detected in areas of tumor infiltration in adipose tissue and in the proximity of small tumor sheets and among some of the sheets. Using polyclonal antibody anti-CD3 on paraffin sections, we also found that the lymphocytic infiltrates consisted mostly of CD3$^+$ T-cells; up to 70% in the invasive ductal carcinoma and up to 80% in the medullary carcinomas. The proportion of CD4$^+$ and CD8$^+$ T-cells established by the monoclonal antibodies OKT4 (anti-CD4) and OKT8 (anti-CD8) in the cell infiltrate varied in the cases of breast cancer with polymorphocellular or lymphocytic cell infiltration. In the atypical medullary carcinoma, CD8$^+$ T-cells predominated approximately twice than CD4$^+$ T-cells. CD8$^+$ T-cells showing cell-to-cell contact with tumor cells were rarely observed but non-apoptotic changes were detected in these tumor cells. Other cases of invasive ductal and atypical medullary carcinomas with predominant lymphocytes in cell infiltrate showed almost equal presentation of CD4$^+$ and CD8$^+$ T-cells. The preponderance of CD4$^+$ T-cells over CD8$^+$ T-cells was also observed.

B-lymphocytes (CD19$^+$ cells), which express membranous marking, were found in 15-30% of tumors with predominant lymphocytes.
The plasmocytes in different types of cell infiltration in the invasive ductal carcinomas were found to produce IgA and IgG with kappa light chain and more rarely with lambda chain. Plasmocytes producing mainly IgM or IgG were also detected. In typical medullary carcinomas among lymphocytic infiltrate, 20-50% of the plasmocytes were found to produce IgM and IgA, while 7-12% of plasmocytes reacted positively to IgG. In peripheral areas of atypical medullary carcinomas, 25-50% of the plasmocytes were marked mainly for IgA (kappa and lambda), but among the infiltrate rich in lymphocytes, the plasmocytes which reacted positively to IgA, IgG or IgM were few.

Macrophages marked for lysozyme were detected among the infiltrates of lymphocytes in the vicinity of tumor sheets. Marked cells were also observed by using a polyclonal antibody for α1-antitrypsin. They were found in the proximity of areas of necrosis and between the cells of infiltrate in the periphery of tumor in the atypical medullary carcinoma.

Morphological changes in the reactive structures of the axillary lymph nodes (reactive changes)

Out of 742 axillary lymph nodes from 144 patients, stromal reaction and the reactive changes were examined in 617 axillary lymph nodes in 112 patients. Changes in the reactive structures (reactive changes) were found in 282 (45.7%) lymph nodes and were missing in 335 (54.3%) lymph nodes. In the remaining 32 cases, only 125 unreacted lymph nodes were detected.

The reactive changes in T- and B-dependent cell regions of lymph nodes were determined by proliferation of immunocompetent T- and B-lymphocytes induced by tumor antigens. The active paracortex showing paracortical hyperplasia was populated by small lymphocytes with high density, scarce large pyroneminophilic cells (immunoblasts), histiocytes and interdigitating cells. Post-capillary venules (PCV's) by mean number from 4.2 to 13.8 in high power field (16 × 40) predominantly with high cuboidal endothelial cells, and lymphocytes in their lumen were found. Paracortex of Grade I was found in 87.6% of T type and of 75.5% in TB type, while paracortex Grade II was established in 12.3% and 24.4%, respectively. Germinal centers of the lymph node follicles in B and mixed types were determined as Grade I and Grade II. Secondary follicles with germinal centers in two rows were also observed. Centroblasts, centrocytes and macrophages, ingested apoptotic bodies, were detected in the germinal centers. The mantle zone of the follicles was better expressed around smaller centers. Sinus histiocytosis was defined from 0 to II degree. The plasmatization of the lymph nodes was graded as 0 and Grade I; as an exception-Grade II.

Lymph nodes of different reactive types and grades were found in each patient. The mixed type most frequently observed was T.B, while both T,B and T,B were few. The availability of lymph nodes of paracortical type (T) together with nodes of TB type was more frequent and more rare T with B or B with TB type. The lymph nodes with reactive changes of T and TB types were observed significantly more frequently than those of B type (p<0.0001 and p<0.0001, respectively; chi-square test). (Table 1) Furthermore, in each type of reactive change, lymph nodes without cancer metastases were more frequently found than those with metastases; they are referred to as small cancer nests (100 µm-400 µm).

The lack of reactive changes in lymph nodes showed absence of reactivity. The following alterations were also detected: hypocellularity (lymphocyte depletion) with a small number of PCVs possessing flattened endothelial cells, atrophy, lipomatosi, fibrosis and hyalinisation. Cancer metastases were established in 63 (18.8%) from 335 lymph nodes with lack of reactive changes. The ratio of reacted lymph nodes to unreacted lymph nodes varied from 2/6 to 5/2. A significant association was observed between the type of cell infiltration in tumors and the type of reactive changes in lymph nodes (Table 2) (p=0.0001; chi-square test). Furthermore, reactive changes of T and TB types were more frequent than those of B type in the lymph nodes in tumors with cell infiltration type of lymphocyte predominant and with scanty or missing cells (Table 2, Figures 1 and 2).

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<th>Table 1. Classification of axillary lymph nodes with reactive changes of breast cancer patients by region and status of metastasis</th>
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<td>Region</td>
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*With small cancer. |
Figure 1. Reactive changes in axillary lymph nodes of breast carcinoma with cell infiltration in which lymphocytes predominated. A. Atypical medullary carcinoma. Cell infiltrate abundant in lymphocytes was disposed between large sheets of tumor cells. B. Groups of tumor cells among the lymphocytic infiltrate without signs of apoptosis. C. Axillary lymph node with reactive changes of mixed type of Grade 1 (T.Ik). Secondary follicle presented by its germinal center (asterisk) appeared in the paracortex, rich in lymphocytes. (H&E, ×100) Breast carcinoma in a 49-year-old woman.

Figure 2. Reactive changes in axillary lymph nodes of breast carcinoma with scanty cell infiltration. A. Invasive ductal carcinoma with trabecular arrangement of tumor cells. Only a few cells are observed in dense fibrous stroma. B. Axillary lymph node showing paracortical activity. Small lymphocytes and numerous PCVs, possessing high cuboidal endothelial cells with vesicular nuclei (arrows), are seen in the paracortex. (H&E, ×100) Breast carcinoma in a 51-year-old woman.
Inhibiting effect of the culture supernatants obtained from stimulated ERFC and cells of the subset T_x and T_y

The proportion (± standard error (SE)) of ERFC separated from PBMC of 94 study subjects was 36.8±3.4%. The proportion (± SE) of CD2+ cells and T cell subsets in ERFC was as follows: 94.7±3.4% for CD2+ cells, 80.9±2.9% for CD3+ T cells, 52.4±2.8% for CD4+ T cells and 41.2±6.3% for CD8+ T cells. The proportion of CD3+CD57+ NK cells in ERFC was 8-9.5%, while that of CD3+CD56+ NK cells in ERFC was less than 5%.

In the T_x subset, 68.7% of the cells were OKT4 (CD4) referred to helper/inducer cells, while 36.4% were OKT8 (CD8)−T cytotoxic/suppressor cells. In the T_y subset, 16.6% of the cells were OKT4 (CD4) and 70.3% were OKT8 (CD8).

The supernatants of ERFC from PBMC, containing predominantly T-lymphocytes stimulated with cancer extract, showed in 70.5% of the tests an inhibition effect against migration of Ehrlich ascites tumor cells, while in 29.5% of the tests, the supernatants showed no inhibition effect (inactive supernatants). The supernatants of stimulated T_x subset showed an inhibition effect in 32 (76.2%) out of 42 patients; the mean (± SE) of Mgl was 0.58±0.08. The mean (± SE) of Mgl in the remaining 10 (23.8%) patients showing no inhibition effect was 1.05±0.14. Supernatants of T_y subset showed no inhibition effect in 40 (95%) out of 42 patients; the mean (± SE) of Mgl was 1.04±0.11. The mean (± SE) of the Mgl of the examined supernatants is shown in Table 3. The effect of inhibition on Ehrlich-cell migration significantly decreased and abrogated (increased Mgl) when active and inactive supernatants of ERFC were mixed or supernatants of T_x and T_y subsets were mixed (Table 3). Supernatants showed no inhibition effect when ERFC from 20 cancer patients were stimulated by extracts of benign breast tissue; the mean (± SE) of Mgl was 0.95±0.06. In 12 patients with fibrocystic disease of the breast, supernatants showed no inhibition effect after stimulation of ERFC with cancer extract (Mgl=0.97±0.10).

Active and inactive supernatants were also obtained by stimulating ERFC and the subsets T_x and T_y, separated from lymphocytes of regional lymph nodes with reactive changes. The means (± SE) of Mgl of the active supernatants, the inactive supernatants and combination of active and inactive supernatants were 0.60±0.08, 1.15±0.07 and 1.11±0.10, respectively. The Mgl of the active supernatants was significantly smaller than that of the combination of active and inactive supernatants (p=0.0001; Mann-Whitney U test).

The means (± SE) of Mgl of the supernatants of T_x and T_y were 0.62±0.13 and 1.05±0.12, respectively.

The histological examination of the axillary lymph nodes revealed similar reactive changes both in occasion of production of inhibitory factor in the active supernatants of stimulated ERFC (from PBMC) and without such a production in inactive supernatants. The most frequent reactivity of lymph nodes was type T1B1, but the following reactive changes were also found: T1B, T2B, T1B. Plasmaticization was of Grade 0 and I. Paracortical activity was found in lymph nodes from which ERFC, separated from their lymphocytes, produced inhibitory factor after stimulation in the active supernatants. Moreover, paracortical hyperplasia (T.B. and T.B.) was similarly detected when production of inhibitory factor lacked in inactive supernatants. Reactivity in lymph nodes of type T.B. was also found when both of T_x and T_y subsets were obtained from ERFC, separated from PBMC and lymph node lymphocytes, and examined for production of inhibitory factor. Other reactive changes in particular lymph nodes were T.B., T.B., T.B. and T.B.

**Discussion**

Accumulation of TIL in carcinoma has been assumed as a result of antitumor immune response, but these cells became functionally inhibited due to the immunosuppressive tumor microenvironment. According to another conception, TIL represents an inflammatory response with consecutive binding to tumor-antigenic system. Preponderance of CD3+ T-cells in TIL in the presence of CD4+ and CD8+ T-cells was found in different breast carcinomas in our cases as was reported previously. Predominance of CD8+ T-cells has been assumed as an attempt for immune reaction against malignant tumor. However, it could be considered the data for reduction of clonogenicity of TIL and poor antitumor cytotoxicity against autologous tumor cells.

The rate of B-lymphocytes in the tumor stroma was smaller than that of T-lymphocytes. B-memory cells, which are recirculating lymphocytes, immigrated in the tumor and differentiated in plasmocytes. The classes of the produced immunoglobulins were reported. Previously, NK cells have been detected in a small number among the cell infiltrate of breast cancer.

Macrophages in carcinoma and their possible role in antitumor cytotoxicity were examined elsewhere.

To assess the reactive changes in lymph nodes we graded the activated T- and B-cell structures and defined three immunoreactive types (T, B and TB). The paracortical type (T) and the mixed type (TB) testified for activation of the T-, T- and B-cell systems. The T- and B-cell areas activated most frequently of Grade I and rarely of Grade II but varied in the lymph nodes. It should be emphasized that

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<th>Table 3. Migration index (mean±SE) by culture supernatants of ERFC separated from PBMC and the subsets T_x and T_y</th>
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<td><strong>Erythrocyte rosette-forming cell (ERFC)</strong></td>
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<td>Active supernatants</td>
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expanded paracortical areas of Grade I (T.) were active (paracortical activity) and did not correspond to paracortex Grade I as described elsewhere.13,15

The reactive changes in lymph nodes of T and TB types with paracortex of Grade I and II were predominant not only for cell infiltration in carcinoma with lymphocyte predominant but also for the scanty one. In this occasion, a blockage of the immigration of immunocompetent cells at the level of the microcirculatory bed of tumor could be suggested.

We showed that inhibitory factor was produced from ERFC (consisting mostly of T-lymphocytes) and from the subset T6, both of which received from PBMC and lymph node lymphocytes, when they were stimulated with cancer extract containing membrane associated with tumor solubilized antigens.14,22

Production of inhibitory factor was revealed from spleen cells of mice stimulated with tumor extract.15,16 The use of Ehrlich ascites cells in the assay of migration inhibition in vitro was based on the nonspecific effect of the inhibitory factor against different tumor (target) cells. Active inhibiting fractions were isolated from crude supernatants in the ranges of molecular weight between 14,000 and 5,800 daltons and under 5,800.16-23 Our findings showed that in addition to active supernatants inactive ones without inhibition effect on Ehrlich-cell migration were obtained from stimulated ERFC separated from PBMC and lymphocytes from axillary lymph nodes showing paracortical activity. Different immunocompetent cell subsets, induced by tumor antigens i.e., CD4+ T-cells (helpers) producing inhibitory factor17 and CD8+ T-cells (cytotoxic and suppressor cells), could be generated and proliferated in lymphoid organs (spleen and lymph nodes), particularly in active paracortex of lymph nodes. The suppressor cells produced in the inactive supernatants suppressor factor, which blocked or abrogated the effect of inhibitory factor in the combination of active and inactive supernatants, possibly by engaging the receptors of tumor cells. Increased suppressor activity in peripheral blood lymphocytes has been found in persons with different cancers.20,26

We have indicated the depressive effect of the inactive supernatants of T6 subset, obtained from ERFC of PBMC, which decreased or abrogated the inhibitory effect of the active supernatants of T6 subset when combined with them. This effect was due to the presence of suppressor factor produced by suppressor cells of T6 subset. When the subset T6 was obtained from T-cells (ERFC) of regional lymph nodes with paracortical activity, the generation and proliferation of suppressor cells producing suppressor factor in the paracortex was obvious. The findings support our hypothesis that in patients with breast carcinoma cancer-extract stimulated ERFC (T-lymphocytes) separated from PBMC and cells of lymph nodes, showing paracortical activity, produced inhibitory factor from helper T-cell subset in active supernatants and suppressor factor from suppressor T-cell subset in the inactive supernatants depressing the effect of inhibitory factor. The integral inhibitory effect of the supernatants obtained from ERFC of peripheral blood and lymph node may be positive or negative.

The suppressor factor, released from suppressor cells in lymph nodes, probably inhibited the helper CD4+ T-cells producing cytokines necessary for differentiating and proliferating CD8+ T cytotoxic lymphocytes, but its direct effect on these lymphocytes was not excluded as well. Since suppressor T-cells (T6 subset) possibly propagated in the active paracortex of regional lymph nodes, the prognostic significance of the paracortical activity was questioned. We also suggested the possibility that suppressor cells, immigrating and accumulating in tumor site, influence cytotoxic lymphocytes by blocking their function.

The possible relationship between inhibitory factor and IL-10 could be suggested. IL-10 inhibits the antigen-specific T-cell proliferation and cytokine production, and alters cell trafficking and migration.24 It also refers to the indirect influence of TGF-β on the production of inhibitory factor and suppressor factor by its effect of inhibiting IL-2 dependent T-cells from proliferating and secreting various cytokines;17 we have, however, no findings in this respect.

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References


