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Characterization of T-lymphocyte subpopulations infiltrating primary breast cancer

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Summary. Characterization of T-lymphocyte subpopulations adjacent to and infiltrating the primary tumor of breast cancer was carried out using a direct immunofluorescence procedure with the antibodies anti-(Leu-2a) for suppressor/cytotoxic ($CD8^+$) and anti-(Leu-3a) for helper/inducer ($CD4^+$) T-lymphocytes. Fifty-six primary malignant tumors with lymphoid infiltration were studied. The majority (58.9%) were infiltrating duct carcinoma. There were metastases to axillary lymph nodes in 6.67% of the patients. Massive lymphoid infiltration (>40 lymphocytes per ×400 microscopic field) was found in 19.6% of the tumors and moderate infiltration (20–40 lymphocytes per field) in 51.8%. In all the tumors studied there was a reversed $CD4^+/CD8^+$ ratio as compared to that found in normal peripheral blood. In 66.1% the $CD4^+/CD8^+$ ratio (helper/suppressor) was less than 1.0. The reversed ratio was due to a significant decrease in the number of helper cells ($P < 0.0005$). The most significant drop was in the stroma area ($P < 0.0001$) as well as in the tumor tissue ($P = 0.001$). Of particular interest was the significant positive correlation between the age of the patients and an increased number of $CD4^+$lymphocytes in the stroma ($P = 0.02$). Significant negative correlations were found between a reduced number of $CD4^+$ lymphocytes or $CD4^+/CD8^+$ ratio and several histological parameters: tumor diameter, pleomorphism, nucleus/cytoplasm ratio. There was also a significant positive correlation between the total number of $CD8^+$ lymphocytes infiltrating the tumor tissue and the number of axillary lymph nodes with metastatic disease ($P = 0.03$). It is suggested that the reversed ratio of $CD4^+/CD8^+$ lymphocytes may significantly affect the host/tumor immune surveillance.

Introduction

Lymphocytic infiltration in the area of and within a malignant tumor of the breast is regarded as evidence of an immunological response to the tumor [12]. Whether this is associated with a better prognosis is still a matter of debate [3, 12, 16]. In a previous retrospective study of 78 women with stage I breast cancer we found a correlation between lymphoid infiltration and a higher rate of recurrence and mortality [2].

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It has long been recognized that patients with advanced cancer show a depression of the cell-mediated immune reaction [6, 27] and alterations have been reported in the subpopulations of T-lymphocytes in the peripheral blood of cancer patients [13, 21].

We present here the results of a study carried out to characterize the lymphocytic infiltration present at or around the site of the breast cancer, including the number and ratio of T-cell subsets.

Materials and methods

During the years 1985 and 1986 we obtained the primary tumors of breast carcinoma from 105 patients who underwent surgical procedures in the Beilinson Medical Center. Multiple histological slides were made from each primary tumor and Giemsa stain was used primarily to identify any lymphoid infiltration in the tumor tissue and its surrounding stroma. Fifty-six tumors were found to have lymphoid infiltration and comprise the study material. The age of the patients ranged from 25 to 83 years (mean: 62.1 years). The clinical stage of the participating patients is shown in Table 1. The surgical procedures undergone were either modified radical mastectomy or quadrantectomy with axillary lymph-node block dissection. The mean diameter of the tumors was 2.8 ± 1.65 cm. Infiltrating duct carcinoma was the diagnosis in 33 (58.9%) of the tumors, whereas 19 patients (33.9%) were diagnosed as having infiltrating duct carcinoma with areas of intraductal carcinoma, and 4 (7.2%) had intraductal carcinoma alone. The mean number of lymph nodes removed from the axilla was 15 ± 6.1, of which 6.67% were infiltrated by the tumor.

Following eosin/hematoxylin staining each tumor was characterized histologically on the basis of the following

Table 1. The distribution of the breast cancer patients studied in regard to TNM and clinical staging

	N_0M_0	N_1M_0
T_1	16	10
T_2	15	12
T_3		3
Stage		
I	16	
II	25	
III	15	

parameters: tumor borders, appearance of nucleus (vacuolar; hyperchromatic), nuclear pleomorphism, mitosis, nucleus/cytoplasm ratio, presence of nucleoli and intensity of lymphoid infiltration near and within the tumor tissue.

The axillary lymph nodes removed were also examined for tumor cell infiltration by routine histological methods.

Additional 4-μm sections were cut from the frozen primary tumor for direct immunofluorescence staining. After the slides had been brought to room temperature they were fixed in cool acetone (4° C). The T-lymphocyte subpopulations were then identified by a direct immunofluorescence procedure, using monoclonal antibodies to $CD4^+$ (helper/inducer) and $CD8^+$ (suppressor/cytotoxic) cells. Anti-(LEU-2a)-fluorescein-isothiocyanate (FITC) and anti-(LEU-3a)-(PE) (Becton Dickinson) were used diluted 1:10 in phosphate-buffered saline. The histological slides were incubated with fluorescent monoclonal antibodies for 1 h in a dark chamber on ice and then washed with phosphate-buffered saline for 3 min. Using a microscope equipped for epifluorescence with standard fluorescein filters, the anti-(LEU-2a)-FITC-labelled cells and the anti-(LEU-3a)-PE-labelled cells were observed simultaneously in the same microscopic field. For each primary tumor the $CD4^+$ and $CD8^+$ lymphocyte subpopulations were counted in 10–15 different high-power fields (×400) at various locations in the stroma and within the tumor tissue. The average number of $CD4^+$ and $CD8^+$ lymphocytes and their ratio were calculated for the different locations, as noted above. Using an indirect immunofluorescence procedure with anti-(LEU-2a) and anti-(LEU-3a) and FITC-labelled anti-(mouse IgG) serum, the T-lymphocyte subpopulations were classified in 10 tumors chosen randomly. Counting similar fields in the adjacent histological slides we obtained results with a high correlation between the two methods.

The statistical package for social science, SPSS version 9, was used to determine the correlation (r) or significance of difference between the different groups. Analysis of variance was also used.

Results

Pattern of lymphocyte infiltration

The histological parameters of the primary tumors and the intensity of lymphoid infiltration adjacent to and within the tumor tissue are shown in Tables 2 and 3, respectively.

Table 3. Intensity of lymphoid infiltration adjacent to and within the tumor tissue of breast carcinoma

Total number of lymphocytes ($CD4^+$ and $CD8^+$) per ×400 microscopic field	Percentage of patients (number of patients)
<20	28.6 (16)
20–40	51.8 (29)
>40	19.6 (11)

In only 12.5% were the tumor borders well defined, whereas most (57.1%) showed elements of invasion of the stroma. The great majority of tumors had vacuolated nuclei (87.5%), a low-to-moderate rate of nuclear pleomorphism (89.3%), and a low-to-moderate rate of mitosis (85.8%); 66.1% of the tumors showed nucleoli. In only 10.7% of the tumors was the nucleus/cytoplasm ratio slightly reversed.

In the majority of the tumors (51.8%) lymphoid infiltration was moderate and in only 19.6% was it massive. There were lymphocytes scattered in the stroma surrounding the tumor in 67.9% of cases and infiltrating the tumor tissue in 60.7% (there were 10 patients belonging to both categories).

Subpopulations of T-lymphocytes infiltrating breast cancer

There was a positive correlation between the number of $CD4^+$ lymphocytes found in the stroma near the tumor tissue and the age of patients ($r = 0.417$, $P = 0.02$) (Fig. 1). The diameter of the tumor was correlated negatively with the $CD4^+$ lymphocyte subpopulation of T-lymphocytes ($r = -0.46$, $P = 0.02$) (Fig. 1). In most cases of massive infiltration by T-lymphocytes, the majority were $CD8^+$ lymphocytes ($P = 0.0005$). We found only one patient with a normal ratio similar to that found in the tissue in benign disease [1]. In 33.9% the ratio was from 3.3 to 1.0 and in 66% it was lower than 0.99 (Table 4). A highly significant correlation was found between the ratio of $CD4^+/CD8^+$ and the total number of $CD4^+$ lymphocytes ($P = 0.00001$). Thus the decrease in the ratio $CD4^+/CD8^+$ resulted from the significant decrease in the number of $CD4^+$ lymphocytes (Fig. 2A) ($P < 0.0005$). This significant decrease was found in the stroma (Fig. 2B) ($P = 0.0001$) as well as within the tumor tissue ($P = 0.001$) (Fig. 2C).

Table 2. Tumor-defining histological parameters in 56 primary tumors of breast carcinoma

Category	Borders[a]		Nucleus type[b]		Pleomorphism[c]		Mitosis[d]		Nucleus/cytoplasm ratio[e]		Presence of nucleolus[f]	
		(%)		(%)		(%)		(%)		(%)		(%)
1	7	12.5	49	87.5	31	55.4	10	17.9	6	10.7	37	66.1
2	32	57.1	7	12.5	19	33.9	38	67.9	31	55.4	19	33.9
3	17	30.4			6	10.7	8	14.3	19	33.9		

[a] Borders: 1, well-defined; 2, ill-defined; 3, marked invasion
[b] Nucleus type: 1, vacuolar; 2, hyperchromatic
[c] Pleomorphism: 1, low-grade; 2, moderate; 3, high-grade
[d] Mitosis: 1, low-grade; 2, moderate; 3, high-grade
[e] Nucleus/cytoplasm ratio: 1, low; 2, moderate; 3, high
[f] Presence of nucleus: 1, none; 2, present

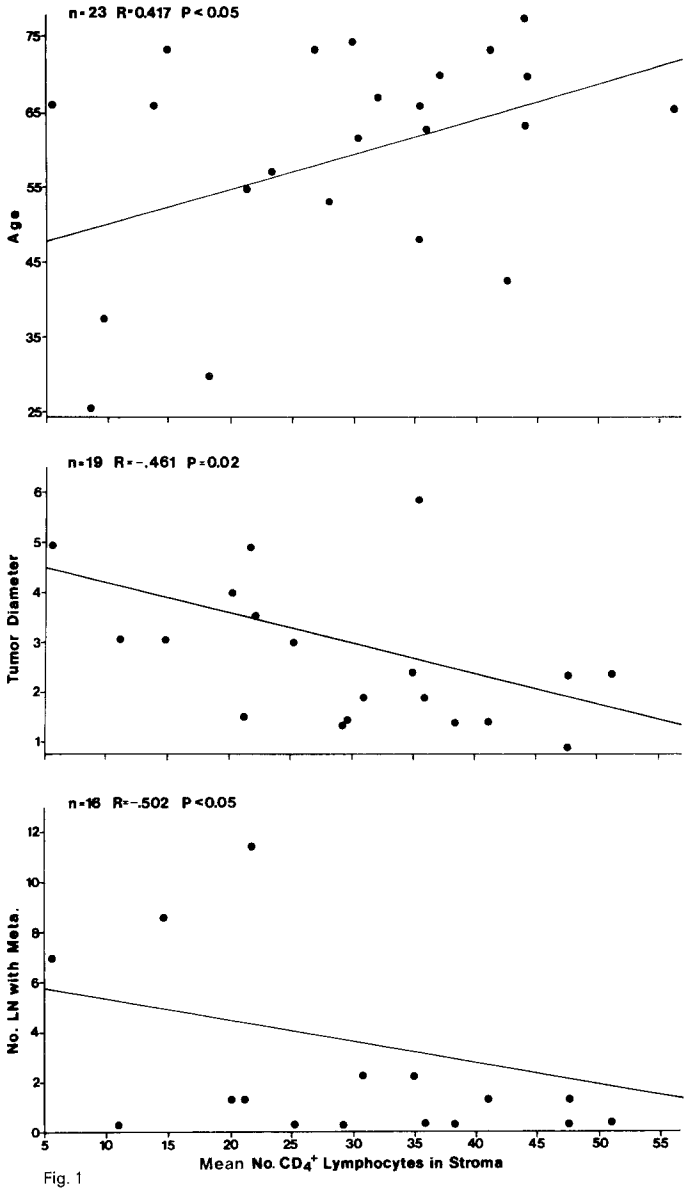

Fig. 1

Fig. 1. The number of $CD4^+$ lymphocytes in the stroma adjacent to the tumor tissue correlated with age of patients, diameter of tumor and number of regional lymph nodes infiltrated by tumor cells. (The variation in numbers of patients is due to missing data)

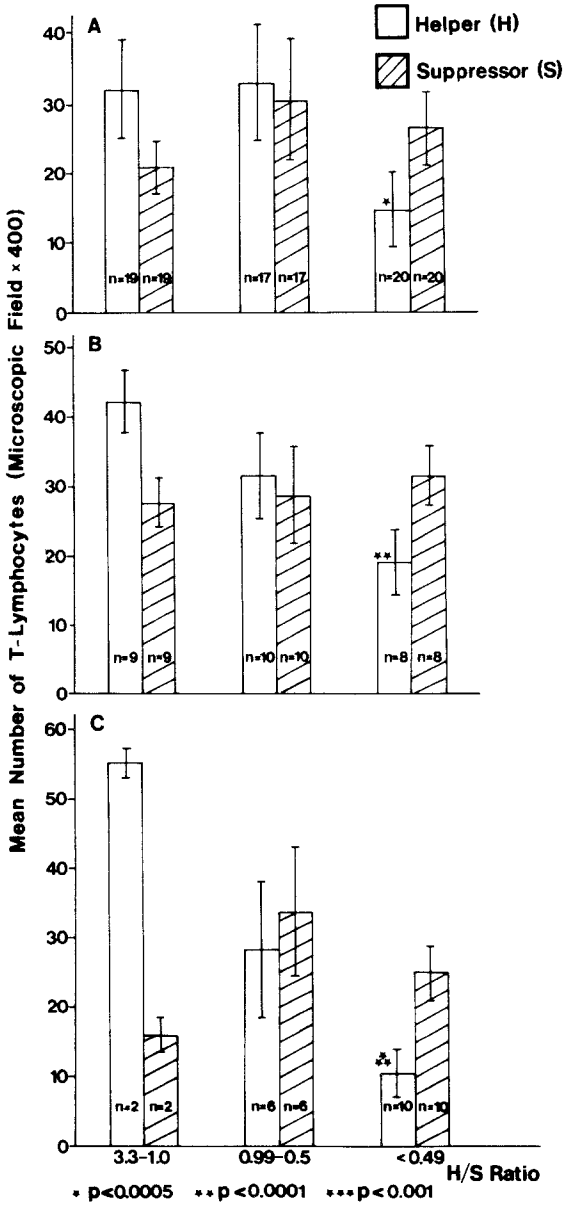

Fig. 2. Mean number of T-lymphocyte subpopulations correlated with the H/S($CD4^+/CD8^+$) ratio in breast cancer: **A** adjacent to and within the tumor tissue; **B** in the stroma surrounding the tumor; **C** infiltrated within the tumor tissue

The ratio of $CD4^+/CD8^+$ was significantly lower with high-grade cell pleomorphism than it was with low and moderate pleomorphism (0.122 versus 0.735, $P = 0.02$). A decrease in the $CD4^+/CD8^+$ ratio in the stroma was also observed when the primary tumor was characterized by a markedly reversed nucleus/cytoplasm ratio ($P = 0.001$).

Table 4. Difference in $CD4^+/CD8^+$ ratios in breast cancer patients

$CD4^+/CD8^+$ ratio	Number of patients
3.30–1.00	19 (33.9%)
0.99–0.50	17 (30.3%)
<0.49	20 (35.8%)

The other histological parameters had no significant correlation with the number or ratio of $CD4^+$ and $CD8^+$ lymphocytes.

The number of lymph nodes infiltrated with tumor cells was positively correlated with an elevated total number of $CD8^+$ and a decreased number of $CD4^+$ lymphocytes in the stroma ($P = 0.05$, $P = 0.01$) (Fig. 1).

Discussion

In this study we analyzed the T-lymphocyte subpopulations infiltrating the primary lesion of breast cancer. The use of frozen sections of the tumor tissue and monoclonal antibodies made it possible to quantify the ratio of $CD4^+/CD8^+$ T-lymphocytes infiltrating the stroma and the tumor tissue in the same histological slide. It was felt that a closer look at these subpopulations would be of in-

terest in view of our previous observation indicating a worse prognosis for stage I breast cancer patients with tumor-infiltrating lymphocytes in the primary breast cancer [2]. It is of note that other workers [2, 18] also reported a high percentage of primary breast cancer patients without lymphoid infiltration (55.1% and 80%, respectively), which is similar to our finding of 46.7%.

A number of recently reported studies deal with T-lymphocytes in breast cancer patients. Some of these [13, 21] studied mainly peripheral blood T-lymphocytes. Others [1, 9, 26] have characterized the tumor-infiltrating lymphocytes in breast cancer. Kaszubowski et al. [13], Dillman et al. [5] and others [11, 14, 17, 23] found a relative and absolute decrease in the number of T-lymphocytes in the peripheral blood of patients with advanced metastatic carcinoma. A similar significant reduction in the total number of T-lymphocytes in the tumor tissue was observed by An et al. [1] in patients with an advanced stage of breast cancer (> stage III) as compared to that found in an early stage and in benign conditions such as fibrocystic disease.

Most previous reports agreed in finding an alteration in the ratio of $CD8^+$ and $CD4^+$ T-lymphocytes in the peripheral blood and in the tumor-infiltrating lymphocyte population. Vose and Moore [25] found a marked elevation in $CD8^+$ T-lymphocytes in the peripheral blood and in the $CD8^+$ lymphocytes extracted from the tumor tissue. Supporting these results is the recent study by Whiteside et al. [26], who found an alteration in the $CD4^+/CD8^+$ ratio, with a predominance of $CD8^+$ over $CD4^+$ in most of the tumor tissue specimens. On the other hand, a predominance of $CD4^+$ in the tumor-infiltrating lymphocyte subpopulations has been reported by Gottlinger et al. [9].

Studies concerning the ratio of $CD4^+$ to $CD8^+$ T-lymphocytes in benign and malignant tumors of the breast have demonstrated a predominance of $CD4^+$ lymphocytes in both benign and malignant breast tumors, but a much lower ratio of $CD4^+/CD8^+$ in the malignant tumors than in the benign tumors (1.79 and 6.8, respectively) [1, 9].

In all of our patients the $CD4^+/CD8^+$ ratio in the tumor tissue was abnormally reduced or reversed. In 66% there was a predominance of $CD8^+$ (Table 4). In analyzing the alteration of $CD4^+/CD8^+$ from the normal range we found a significant reduction in the total number of $CD4^+$ lymphocytes ($P < 0.0005$). This significant reduction was found in different areas – the stroma adjacent to the tumor tissue and within the tumor tissue ($P < 0.0001$ and $P < 0.001$, respectively).

Unlike others [1] we found a significant negative correlation between the number of $CD4^+$ lymphocytes or the $CD4^+/CD8^+$ ratio and some of the histological parameters – tumor diameter, cell pleomorphism, nucleus/cytoplasm ratio, and increased number of metastases in regional lymph nodes. All these histological parameters can determine the immaturity of the tumor and the state of advanced tumor diesease.

Elderly patients, mainly post-menopausal, are considered to have a better prognosis than younger patients. In this respect it was interesting to find a significant positive correlation between the age of patients and the number of $CD4^+$ lymphocytes in the stroma near the primary tumor tissue.

The tumor-infiltrating lymphocyte population in breast cancer represents an immunological host reaction [24]. This immune response is made ineffective by various immunological suppressive mechanisms. In their in vitro studies Vose and Moore [25] demonstrated the presence of suppressor activity capable of reducing immunological responses. The reduced number of $CD4^+$ lymphocytes and the abnormality of the ratio of $CD4^+/CD8^+$, as found by us and others [1], may be an expression of a suppressive mechanism involving substances blocking T-lymphocyte replication, secreted by the tumor cells [4, 7, 8, 25]. This hypothesis is supported by the results of Whiteside et al. [26], who stressed the relatively low number of activated T-cells (10%), as expressed by surface antigen to interleukin II. Low levels of interleukin II, resulting from a reduced number of $CD4^+$ lymphocytes, have been found to interfere with the generation of LAK cells, which play a major role in lysis of tumor cells [10, 15, 19, 20].

Additional studies concerning the tumor-infiltrating lymphocytes are required to clarify further the relationship between their subpopulations in breast cancer, with the aim of enhancing their cytolytic function against the tumor cells.

References

1. An T, Sood U, Pietruuk T, Cummings G, Hashimoto K, Crissman J (1987) In situ quantitation of inflammatory mononuclear cells in ductal infiltrating breast carcinoma. Am J Pathol 128: 52
2. Bilik R, Mor C, Wolloch Y, Dintsman M (1986) Histopathological high risk factors influencing the prognosis of patients with early breast cancer. Am J Surg 151: 460
3. Bloom HJ, Richardson WW, Field JR (1970) Host resistance and survival in carcinoma of breast: a study of 104 cases of medullary carcinoma in a series of 1,411 cases of breast cancer followed for 20 years. Br Med J 1: 181
4. Cantor H, Boyse EA (1975) Functional subclasses of T-lymphocytes bearing different Ly antigens. J Exp Med 141: 1376
5. Dillman RO, Koziol JA, Zavanelli MI, Royston I (1984) Immunoincompetence in cancer patients. Cancer 53: 1484
6. Eilber FR, Morton DL (1970) Impaired immunologic reactivity and recurrence following cancer surgery. Cancer 25: 362
7. Engleman EG, Benike CJ, Glickman E, Evans RL (1981) Antibodies to membrane structures that distinguish suppressor/cytotoxic and helper T lymphocyte subpopulations block the mixed leukocyte reaction in man. J Exp Med 153: 193
8. Evans RL, Wall DW, Platsoucas CD, Siegal FP, Pikning SH, Testa CM, Good RA (1981) Thymus-dependent membrane antigens in man: inhibition of cell-mediated lympholysis by monoclonal antibodies to TH2 antigen. Proc Natl Acad Sci USA 78: 544
9. Gottlinger HG, Rieber P, Gokel JM, Lohe KL, Riethmuller G (1985) Infiltrating mononuclear cells in human breast carcinoma: predominance of T4 monocytic cells in the tumor stroma. Int J Cancer 35: 199
10. Grimm EA, Ramsey KM, Mazumder A, Wilson DJ, Djeu JY, Rosenberg SA (1983) Lymphokine-activated killer cell phenomenon. J Exp Med 157: 884
11. Gross RL, Latty A, Williams EA, Newberne PM (1975) Abnormal spontaneous rosette formation and rosette inhibition in lung carcinoma. N Engl J Med 292: 169
12. Hamlin IM (1968) Possible host resistance in carcinoma of the breast: a histological study. Br J Cancer 22: 383
13. Kaszubowski PA, Husby G, Tung KSK, Williams RCG (1980) T-lymphocyte subpopulations in peripheral and tissues of cancer patients. Cancer Res 40: 4648
14. Keller SE, Iochim HL, Pearse T, Siletti DM (1976) Decreased T-lymphocytes in patients with mammary cancer. Am J Clin Pathol 65: 445

15. Morgan Da, Ruscetti FW, Gallo R (1976) Selective in vitro growth of T lymphocytes from normal human bone marrows. Science 193: 1007
16. Morrison AS, Black MM, Lowe CR (1973) Some international differences in histology and survival in breast cancer. Int J Cancer 11: 261
17. Nemoto T, Han T, Minowada J, Angkur V, Rao TJ (1974) Cell mediated immune status of breast cancer patients: evaluation by skin tests, lymphocyte stimulation and counts of rosette forming cells. J Natl Cancer Inst 53: 641
18. Rosen PP, Saigo PE, Braun DW, Weathers E, DePalo A (1981) Predictors of recurrence in stage I ($T_1N_oM_o$) breast carcinoma. Ann Surg 193: 15
19. Rosenberg SA (1985) Lymphokine-activated killer cells: a new approach to immunotherapy of cancer. J Natl Cancer Inst 75: 595
20. Rosenberg SA, Lotze MT, Muul LM, Reichert CM (1985) Observation on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. N Engl J Med 313: 1487
21. Schroff RW, Gale RP, Fahey JL (1982) In: Vitetta E, Fox CF (eds) B and T cell tumors: biological and clinical aspects. Academic Press, New York, p 285
22. Schroff RW, Gottlieb MS, Prince HE, Chai LL, Fahey JL (1983) Immunological studies of homosexual men with immunodeficiency and Kaposi's sarcoma. Clin Immunol Immunopathol 27: 300
23. Sen L, Borella L (1974) Immunological rebound after cessation of long-term chemotherapy in acute lymphocytic leukaemia: changes in distribution of T and B cell population in bone marrow and peripheral blood. Br J Haematol 27: 477
24. Shimokawara I, Imamura M, Yamanaka N (1982) Identification of lymphocyte subpopulations in human breast cancer tissue and its significance. Cancer 49: 1456
25. Vose BM, Moore M (1979) Suppressor cell activity of lymphocytes infiltrating human lung and breast tumors. Int J Cancer 24: 579
26. Whiteside TL, Miescher S, Hurlimann J, Moretta L, Fliedner V (1986) Clonal analysis and in situ characterization of lymphocytes infiltrating human breast carcinomas. Cancer Immunol Immunother 23: 170
27. Whittaker MG, Rees K, Clark CG (1971) Reduced lymphocyte transformation in breast cancer. Lancet 1: 892

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