

## Clonal analysis and in situ characterization of lymphocytes infiltrating human breast carcinomas

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**Summary.** T lymphocytes were isolated from tumor biopsies in 13 patients with breast carcinomas. Immunohistology with monoclonal antibodies confirmed the presence of mononuclear cell infiltrates composed primarily of T lymphocytes in all tumors studied. While the proportion of T lymphocytes expressing the T4 or the T8 surface marker varied from tumor to tumor as determined by morphometric analysis, T8+ cells were more numerous than T4+ cells in 8/12 breast tumors studied. Relatively few T cells (<10% in 11/12 tumors) were in an activated state as judged by the surface expression of HLA-DR antigens or the receptor for interleukin-2 (IL-2). In 1 case 20% of the infiltrating mononuclear cells were expressing the IL-2 receptor. The tumor infiltrating lymphocytes (TIL) recovered from 10 tumors were cloned in a microculture system that permits proliferation of nearly 100% of normal peripheral blood T lymphocytes (PBL-T). In contrast to normal and autologous PBL-T, frequencies of proliferating T lymphocyte precursors (PTL-P) were depressed (<0.01) in 7/10 TIL preparations indicating a decreased responsiveness of TIL to phytohemagglutinin at the single-cell level. The frequency of PTL-P was noticeably higher in 2 cases (0.03 and 0.09) and close to normal in 1 case (0.39).

A total of 170 clones were expanded in vitro and analyzed for different functional capabilities. Most of these clones expressed the T4+/T8-phenotype (73%) and strikingly 53% of these T4+/T8- clones were cytolytic in a lectin-dependent assay, a functional subset which is uncommon among normal PBL-T. Some clones (10%) lysed allogeneic breast tumor cells (MCF7). Only 15% of the clones displayed natural killer activity. Among the cytolytic clones, 17 of 31 tested were also IL-2 producers irrespective of the T4 or T8 phenotype. Our results show that human mammary carcinomas contain many infiltrating T cells with cytolytic potential. Interestingly, among the proliferating cytolytic T cell clones (56% of the microcultures), many expressed the T4+/T8- phenotype. These findings may indicate that the in situ cytolytic reaction (against unknown antigens) is associated preferentially with class II antigens.

### Introduction

Patients with breast cancer have been reported to have impairments in cell-mediated immunity [16, 17, 21, 22, 33]. In general, however, quantitative and functional abnormalities of peripheral blood lymphocytes (PBL) that have been reported bear no relationship to the extent of disease present, prognosis, or response to therapy. Many mammary carcinomas are infiltrated by mononuclear cells, and the presence of these infiltrates has been interpreted as a favorable prognostic sign [9, 11]. In other studies, however, the prognostic significance of tumor infiltrates could not be confirmed [6]. Immunohistology with monoclonal antibodies (MoAbs) specific for the surface antigens on leukocytes indicated that the mononuclear cell infiltrates present in human breast tumors are composed primarily of T lymphocytes [2, 13, 31]. Others have reported a distinct preponderance of monocytes/macrophages among the leukocytes infiltrating the tumor stroma [10].

Little is known about functional properties of breast tumor-infiltrating lymphocytes (TIL), and their precise antitumor role is not at all understood. Difficulties associated with analysis of cytolytic effector functions in the TIL populations may relate to insufficient cell numbers, to low frequencies of effector cells in populations analyzed, or to the presence of putative inhibitory factors released by tumor and/or other cells.

In this report, we isolated mononuclear cells resident in human breast tumors by enzyme dispersion (as described previously by Vose et al. [36]) and studied TIL in a limiting-dilution assay. This assay allows the determination of the frequency of proliferating T lymphocyte precursors (PTL-P) and the clonal expansion of nearly every normal resting peripheral T lymphocyte [27]. Moreover T cell clones obtained from TIL were subjected to clonal analysis in order to delineate some of the functional properties of the infiltrating T cells. The results were correlated with immunohistochemical data obtained from the same biopsies that were processed for the isolation of TIL.

We showed that T lymphocytes recovered from breast tumors often had lower cloning frequencies than autologous or normal PBL-T in the phytohemagglutinin (PHA)-dependent microculture system we used. There was a high incidence of cytolytic T cell clones among the growing microcultures. More importantly, clonal analysis indicated that a large proportion of these cytolytic clones expressed the T4+/T8- phenotype and many produced interleukin-2 (IL-2).

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## Material and methods

**Breast tumors.** Surgical biopsies were obtained from 14 patients undergoing surgery for a lump in the breast. Of the carcinomas, 12 were infiltrative of the ductal type, 1 was medullary and 1 lobular. Tissues from 13 women (aged from 47 to 86 years) and 1 man (65 years old) were studied. For separation of TIL a piece of each breast tumor (mean weight = 2.3 g; range = 0.6–9.5 g) was collected into sterile culture medium and processed within 2–4 h after surgery. For immunohistology, pieces of tissue (35 to 63 mm<sup>2</sup>) were embedded in OCT compound and snap frozen in isopentane precooled in liquid nitrogen. The tissue was stored at –80 °C until the time of sectioning.

**Immunohistology.** Serial sections 6 µm thick were cut using a cryostat, air-dried for 2 h at room temperature, fixed for 10 min in cold acetone, and kept at –20 °C until immunohistological processing. On the day of processing, the sections were fixed in acetone for 10 min and rehydrated in Tris-buffered saline (TBS). All the procedures were carried out at room temperature. The normal serum and the antisera were diluted in TBS containing 1% gelatin.

The sections were first incubated for 10 min with normal rabbit serum diluted 1/30 and successively for 30 min with each of the following reagents: mouse MoAb diluted 1/30, rabbit anti-mouse Ig diluted 1/60, and peroxidase anti peroxidase (PAP) from mouse diluted 1/80. Finally, all the sections were washed in TBS and stained for 10 min in the diaminobenzidine substrate (75 mg 3–3' diaminobenzidine in 100 ml TBS containing 32 µl H<sub>2</sub>O<sub>2</sub>). Nuclei were weakly stained with hematoxylin. The optimal dilutions of all antisera were determined on sections of lymph nodes and tonsils. In control slides, the first antiserum was replaced by a supernatant of mouse myeloma culture diluted 1/30.

**Monoclonal antibodies.** The following MoAbs were purchased from Becton-Dickinson, Inc., Sunnyvale, Calif. USA: Leu-3a, Leu-11, Leu-7, Leu-1 and MoAb to IL-2 receptor (IL-2 R). MoAb OKT8, OKIa and OKM1 were from Ortho Diagnostic Systems, Raritan, NJ, USA. MoAb MAS 1532 (anti-HLA class I) was from Seralab Ltd., Crawley Down, Sussex, England. Finally, pan B MoAb was from Dakopatts, Copenhagen, Denmark. Monoclonal mouse PAP and rabbit antiserum for mouse immunoglobulins were obtained from Sternberger-Meyer Immunochemicals, Inc., Jarrettsville, USA.

**Microscopic analysis.** Serial frozen sections, each stained with a different MoAb, were reviewed. Stained cells in the stroma and in the tumor mass were observed. All the cells present in the sections were counted. The sections varied in size between 35 and 63 mm<sup>2</sup>. The counts were adjusted to 50 mm<sup>2</sup> when necessary. The mononuclear cell infiltrate was graded as ± (1 to 200 cells/50 mm<sup>2</sup>); + (200 to 700 cells/50 mm<sup>2</sup>) ++ (700 to 4000 cells/50 mm<sup>2</sup>) and +++ (>4000 cells/50 mm<sup>2</sup>).

**TIL preparations.** The TIL-enriched preparations were obtained from suspensions of mechanically and enzymatically disaggregated tumors as described previously [32, 41]. The TIL were then separated from tumor and tissue cells on discontinuous gradients of Ficoll-Hypaque as described by Vose [36]. All TIL-enriched fractions were ex-

tensively washed and incubated in growth medium at 37 °C and in 5% atmosphere of CO<sub>2</sub> for 2–12 h prior to functional assays. The viability of TIL was determined using trypan blue. Lymphocyte numbers in the TIL fractions were determined from May-Grünwald Giemsa (MGG) smears, and T11+ lymphocytes were quantitated by immunofluorescence.

**Peripheral blood and tonsil lymphocytes.** Blood samples (20 ml) were obtained from 10 patients with breast cancer 1 or 2 day after surgery. Heparinized blood was diluted with equal volumes of medium and centrifuged over a cushion of Ficoll-Hypaque according to a standard protocol [3]. Mononuclear cells were harvested, washed, and either used in functional assays or cryopreserved. Normal peripheral blood was obtained from random blood donors at the blood bank. In control experiments, normal PBL were treated with collagenase-DNase mixture for 2 h or overnight under the same conditions used for disaggregation of tumors. Tonsils were obtained from surgery and processed immediately. They were sliced with scalpels, squeezed through metal sieves, and digested with enzymes exactly like TIL. Enzyme-treated, washed, normal PBL and tonsil lymphocytes were used as controls in the functional assays.

All cell washes were performed in sterile RPMI 1640 medium (Seromed, Basel) containing 10% fetal calf serum (FCS) and supplemented with 200 mM glutamine and antibiotics (penicillin, streptomycin, and gentamicin).

**Immunofluorescence.** Fluorescein-labeled MoAb T11 (Coulter Electronics, Inc., Haleah, Fla.) and IL-2 R (Becton Dickinson, Inc., Sunnyvale, Calif) were used to determine numbers of T11+ cells and cells expressing the IL-2 R, respectively, in the TIL and PBL preparations. For double staining of T lymphocytes combinations of phycoerythrin-labeled MoAb IL-2 R or HLA-DR and fluorescein-labeled MoAb T11 were used. About 50,000 cells in 50 µl of medium were placed in 5 × 35 mm plastic tubes (Milan Inst. SA, Geneva) and incubated with 5 µl of MoAb or MoAb mixture for 10 min at room temperature. The cells were washed 3 times in phosphate-buffered saline (PBS) solution containing 0.2% bovine serum albumin and 0.2% sodium azide, fixed briefly in 2% paraformaldehyde solution and cytocentrifuged. Air-dried cytopins were mounted in PBS glycerol and examined under a Zeiss fluorescence microscope. From 100 to 200 cells were counted, and the proportion of fluorescent or doubly-stained cells determined.

**Microcultures of TIL and PBL.** A limiting-dilution microculture method was used [27]. Briefly, cells were seeded in limiting numbers (based on % T11+ cells) in round-bottomed microwells (Costar, Flacon Plastics, Basel) containing irradiated (5,000 R) allogeneic spleen cells (5 × 10<sup>4</sup>) as feeders in a final volume of 0.2 ml RPMI 1640 supplemented with 20% FCS (Amimed, Basel). Allogeneic human spleen cells were obtained from spleens of accident victims. They were cryopreserved in 10% dimethyl sulfoxide and 20% FCS and defrosted as needed. At the onset of culture, PHA (1% v/v) was added to the wells. After 48 h of culture, IL-2-containing supernatant (50% v/v) was added. Microcultures were grown in a humidified atmosphere of 5% CO<sub>2</sub> in air. They were supplemented weekly with

$5 \times 10^4$  irradiated feeder cells suspended in 100  $\mu$ l of medium with IL-2-containing supernatant. Control wells contained irradiated feeder cells which were initially stimulated with PHA and cultured with IL-2-containing supernatants. After 14–25 days of culture each well was scored microscopically for growth.

*Determination of the frequency of proliferating T cells.* Minimal estimates of the PTL-P were obtained by the minimum  $\chi^2$  method from the Poisson distribution relationship between the responding cell number and the logarithm of the percentage of nonresponding (negative) microcultures [34].

*Production of IL-2-containing supernatant.* Supernatants containing T cell growth factor were prepared from human spleen cells. These supernatants were partially purified by precipitations with ammonium sulfate as described by Moretta et al. [25]. Their growth promoting activity was determined using an IL-2-dependent mouse cell line (CTLL, obtained from Dr. H. R. MacDonald, Epalinges) and colorimetric hexosaminidase assay [20].

*Assays of cytolytic activity.* Cytolytic activities of individual microcultures obtained from TIL or PBL plated under limiting-dilution conditions were tested against different types of  $^{51}\text{Cr}$ -labeled target cells: (a) P815 murine mastocytoma tumor cells in the presence and absence of 1% (v/v) PHA i.e., lectin-dependent cell cytotoxicity (LDCC) which allows the detection of cytolytic activity irrespective of the antigenic specificity of the effector cells; (b) K562 human erythroleukemia cells which allows the detection of natural killer (NK)-like activity; (c) MCF7 human tumor breast line was obtained from Dr. H. Diggelmann, ISREC, Epalinges. Cytolytic activity was assayed as described by Moretta et al. [27]. Briefly, cells in each well were resuspended with a micropipette and then divided into two 100  $\mu$ l aliquots. One aliquot was used to measure cytotoxicity; the other was retained in the original well and supplied with IL-2 to allow further growth. The split microcultures were examined microscopically and again divided. Only well-growing microcultures were used for assays of cytolytic activity, and they were repeatedly divided as described above until all assays were completed. Target cells labeled with  $^{51}\text{Cr}$  were placed in wells of V-bottomed microtiter trays ( $5 \times 10^3$ /well). The lymphocyte suspension was added to each well, and the final volume per well was 200  $\mu$ l. In the LDCC assay, PHA was added to the  $^{51}\text{Cr}$ -labeled P815 cells immediately before placing them in microwells (to avoid the formation of cell clumps). The MCF7 cell suspension was prepared using trypsin-EDTA (Gibco). Following trypsinization, the cells were washed 3 times, incubated in medium for 1 h and then labeled with  $^{51}\text{Cr}$  to be used as targets in cytotoxicity assays. Microplates with target cell-lymphocyte mixtures were centrifuged at 100 g for 5 min and then incubated for 4 h at 37 °C. The plates were centrifuged again (200 g for 5 min), and 100  $\mu$ l of supernatant was removed for measurement of  $^{51}\text{Cr}$  release. Specific lysis was calculated according to the formula:

$$\text{Percent specific lysis} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100$$

Spontaneous release was determined in control microcultures containing  $^{51}\text{Cr}$ -labeled target cells and culture medium, but no responder cells, and incubated exactly like experimental microcultures. Maximum release was determined by lysing  $^{51}\text{Cr}$ -labeled cells with 0.1 N HCl. Cultures in which  $^{51}\text{Cr}$  release exceeded the mean spontaneous release by more than 3 SD were considered positive for cytolytic activity.

*Assay for IL-2 Production.* Induction of IL-2 production in microcultures derived from TIL was obtained by PHA stimulation as described by Moretta et al. [24]. Briefly, aliquots of lymphocytes (100  $\mu$ l) derived from each microculture were placed in wells of a U-bottomed microplate. Each well was washed 3 times with 100  $\mu$ l of culture medium, the supernatants were decanted after the last centrifugation step, and cells resuspended in 200  $\mu$ l of culture medium containing 1% (v/v) PHA. Cultures were incubated for 24 h at 37 °C, then 100  $\mu$ l of supernatant from each microculture was removed and added to CTLL indicator cells (obtained from Dr. H. R. MacDonald) at a concentration of  $4 \times 10^3$  per well and IL-2 activity was assessed by a colorimetric hexosaminidase assay [20]. As negative controls, supernatants of irradiated spleen feeder cells (cultured as control microcultures simultaneously with lymphocyte microcultures, washed 3 times, and incubated with 1% PHA for 24 h) were used. Cultures were regarded as positive for IL-2 production when exceeding by more than 3SD the mean optical density at 405 nm of negative control supernatants.

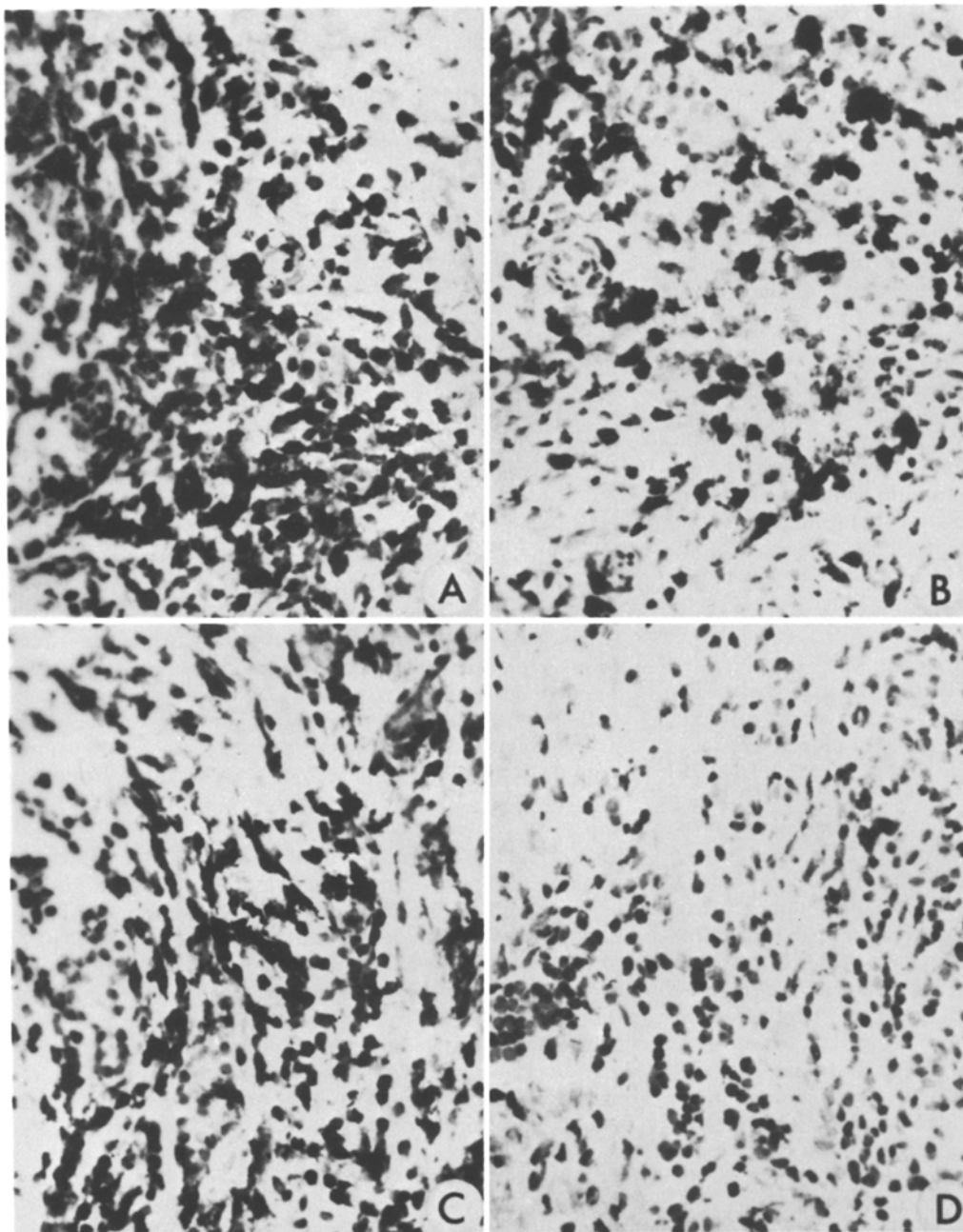
*Phenotyping of microcultures.* Cells obtained from 100  $\mu$ l aliquots of microcultures were washed in medium and incubated with 10  $\mu$ l of fluorescein-labeled monoclonal anti-Leu-2  $\alpha$  (anti-cytotoxic/suppressor) antibody and 10  $\mu$ l of phycoerythrin-labeled monoclonal anti-Leu 3a (anti-helper/inducer) antibody (Becton-Dickinson) for 15 min at room temperature. The cells were washed 3 times in PBS and slides were prepared for immunofluorescence as described above. Each microculture was examined for the presence of Leu-2  $\alpha$ +, Leu 3a+ and doubly-stained cells using a Zeiss microscope.

## Results

### Immunohistology

Immunoperoxidase staining with MoAbs was performed on cryostat-cut sections in 12 of 14 tumors. Mononuclear cells were localized mainly in the tumor stroma (Fig. 1) and were often numerous at the border between normal and malignant tissue. As Table I shows, T lymphocytes were the most prominent component of the infiltrates; B cells (B1+) and monocytes (OKM1+) were infrequent (5%–10% of the infiltrate) and NK cells (Leu-7+ and Leu- were absent. As judged by the expression of the (IL-2R), few (<10%) TIL-T were in the activated state (Fig. 1). Analysis of DR positive T cells was difficult because of the strong reactivity of stromal cells with anti-DR MoAb. It appeared, however, that there were more lymphocytes positive for DR antigens than for the IL-2 R.

The T4/T8 ratios varied from tumor to tumor (Table I), although in 8/12 breast tumors studied, T8+ lymphocytes were more numerous than T4+ lymphocytes. Although only 12 tumors were examined, there seemed to



**Fig. 1.** Infiltrating ductal carcinoma (case 11, D. R.) immunoperoxidase staining with mouse MoAb Leu 3a (A), mouse MoAb OKT8 (B), mouse MoAb OKIa (C) and mouse MoAb IL2R (D). The mononuclear cells positive for each antiserum appear black. The helper T cells (Leu 3a+) are predominant. There are no cells positive for IL-2 R (activated T cells)

be a correlation between the expression of the class I HLA antigens on tumor cells and intensity of lymphocytic infiltrates: tumors positive for class I HLA antigens were the most intensely infiltrated by T lymphocytes.

#### *Preparations enriched in TIL*

Using a combination of mechanical and enzymatic digestion, TIL were recovered from 13/14 breast carcinomas studied. Only tumors containing lymphocytic infiltrates, as determined by examinations of frozen sections at the time of surgery, were selected for this study. The recovery of TIL was variable ranging from  $6 \times 10^3$  to  $7.0 \times 10^6$  T11+ lymphocytes/g of wet tumor tissue. Viability of TIL re-

covered from breast tumors was consistently  $>90\%$  as determined with trypan blue dye. The TIL-enriched fractions were examined for cell morphology on cytocentrifuge smears stained with MGG and found to contain:  $66.4\% \pm 22$  lymphocytes;  $2\% \pm 2.2$  monocytes/macrophages;  $1.2\% \pm 1.1$  granulocytes; rare basophils;  $30\% \pm 23$  tumor, and other nonhematopoietic tissue cells (means of 13 TIL preparations  $\pm$ SD).

Like the in situ analysis, T lymphocytes were the major lymphocyte population ( $30\% - 95\%$  T11+ cells), while B1+ cells and plasma cells were a minor component ( $1\% - 5\%$ ). By morphology, T lymphocytes recovered from breast tumors were small cells which did not express the

**Table 1.** Histopathological diagnoses and immunohistology of human breast carcinomas studied

Histopathological diagnosis		Immunohistology						Ratio T4/T8	IL-2 R+ %
		T11+	T4+	T8+	M1+	B1+	(cell counts in tumor stroma per 50 mm <sup>2</sup> <sup>a</sup> )		
<i>Patients without axillary metastasis:</i>									
1. M. V.	Infiltrating ductal CA,	1040	600	360	52	40	1.67	4.0	
2. B. P.	Infiltrating ductal CA,	190	144	64	0	0	2.25	20.0	
3. C. G.	Infiltrating ductal CA,	1120	378	739	0	10	0.51	3.0	
4. C. E.	Infiltrating ductal CA,	2360	900	1440	125	25	0.63	1.0	
<i>Patients with axillary metastasis</i>									
1. K. J.	Infiltrating ductal CA,	145	49	90	0	36	0.55	0	
2. P. M.	Infiltrating ductal CA,	1710	630	1170	0	57	0.54	0	
3. L. B.	Medullary CA,	5100	2085	2800	0	240	0.74	9	
4. G. A.	Infiltrating ductal CA,	953	130	754	0	18	0.17	0	
5. M. L.	Infiltrating lobular CA,	160	45	109	0	16	0.41	2	
6. B. J.	Infiltrating ductal CA,	185	70	136	14	22	0.51	7	
7. D. R.	Infiltrating ductal CA,	2140	1488	620	20	126	2.4	2.0	
8. G. L.	Infiltrating ductal CA,	1290	986	319	7	15	3.1	2.0	

<sup>a</sup> Positively stained cells were counted in serial sections as described in *Materials and methods*

**Table 2.** Relationship between the in situ infiltration as determined by immunohistology and the recovery of T lymphocytes from human breast carcinomas

Tumor	Immunohistology		Recovered lymphocyte-enriched fraction <sup>a</sup>	
	No. T11+ cells/50 mm <sup>2</sup>	Degree of infiltration <sup>b</sup>	No. TIL/g tumor	No. T11+ cells/g tumor
L. B.	5100	+++	$7.6 \times 10^6$	$7.0 \times 10^6$
C. E.	2360	++	$1.0 \times 10^6$	$4.5 \times 10^5$
D. R.	2140	++	$0.8 \times 10^6$	$3.0 \times 10^5$
P. M.	1710	++	$4.0 \times 10^5$	$2.8 \times 10^5$
G. L.	1290	++	$2.7 \times 10^6$	$1.4 \times 10^6$
C. G.	1120	++	$3.2 \times 10^6$	$1.3 \times 10^6$
M. V.	1040	++	ND	ND
G. A.	953	++	$4.6 \times 10^5$	$3.8 \times 10^5$
			mean $\pm$ SD $\times 10^5$	$6.8 \pm 5.1$
B. P.	190	$\pm$	$6.0 \times 10^4$	$6.0 \times 10^3$
B. J.	185	$\pm$	$1.7 \times 10^6$	$1.1 \times 10^6$
M. L.	160	$\pm$	$2.5 \times 10^5$	$2.0 \times 10^5$
K. J.	145	$\pm$	$1.2 \times 10^6$	$4.0 \times 10^5$
			mean $\pm$ SD $\times 10^5$	$4.3 \pm 4.7$

<sup>a</sup> Biopsies of breast tumors were disaggregated with enzymes and cell suspensions separated as described in *Materials and methods*. The TIL were counted in a hemocytometer in the presence of trypan blue. The percentage of T11+ cells in each TIL-enriched fraction was determined by direct immunofluorescence

<sup>b</sup> Degree of infiltration of the tumors by T lymphocytes was determined as described in *Materials and methods*

receptor for IL-2 or HLA-DR antigens as determined by double immunofluorescence. Occasional large granular lymphocytes were present in TIL. The T4/T8 ratios determined in three TIL preparations were 1.0, 0.8, and 3.0.

#### *Correlation between cells counted in situ and recovered from tumors*

Numbers of T lymphocytes recovered from tumors were compared to those counted in immunoperoxidase sections. While there was no relationship between in situ counts and the T cell recovery in individual cases, as a group, tumors which were densely infiltrated in situ tended to yield more T11+ lymphocytes per gram tumor weight than tumors with scarce infiltrates (Table 2).

#### *Limiting-dilution analysis (Table 3)*

The TIL recovered from 10 breast tumors were initially plated according to the number of T11+ lymphocytes at 0.5, 1, 2, 5, 10, and 100 cells per well. Cell growth was determined microscopically in the individual wells between days 14 and 25 in culture. The frequency of PTL-P was then calculated according to Taswell [34]. In 7 out of 10 patients the PTL-P was markedly decreased with less than 1/100 responding T cells. In 2 cases the frequency of PTL-P was noticeably higher with 1/30 and 1/11 responding T cells and in 1 case the frequency was close to normal (1/2–1/3 responding cells, patient C. E.).

These frequencies were in marked contrast to normal PBL where the frequency of PTL-P ranged from 0.56–1.11

**Table 3.** Frequency of PTL-P Among TIL recovered from tumors and PBL of patients with breast cancer

Tumor	Frequency (95% confidence limits) <sup>a</sup>	
	TIL	PBL
1. B. P.	<0.01 <sup>b</sup>	ND
2. K. J.	<0.01	ND
3. D. A.	<0.01	ND
4. S. A. M.	<0.01	0.05 (0.03–0.07)
5. P. M.	<0.01	0.36 (0.28–0.44)
6. G. A.	<0.01	0.48 (0.38–0.58)
7. M. L.	<0.01	0.05 (0.03–0.07)
8. B. J.	0.03 (0.01–0.03)	0.11 (0.08–0.14)
9. G. L.	0.09 (0.06–0.013)	0.31 (0.24–0.37)
10. C. E.	(A) 0.16 (0.14–0.18) <sup>d</sup> (B) 0.39 (0.28–0.49)	ND
Controls:	Normal PBL	1. 0.56 (0.44–0.68)
		2. 1.10 (0.80–1.41)
		3. 0.86 (0.61–1.11)
	Normal PBL + enzyme <sup>c</sup>	1. 0.53 (0.38–0.66)
		2. 0.47 (0.31–0.63)
	Tonsils	1. 0.63 (0.53–0.75)
		2. 0.76 (0.48–1.04)
	Tonsils + enzyme <sup>c</sup>	1. 0.79 (0.50–1.11)

<sup>a</sup> Frequencies of PTL-P were calculated as described in *Materials and methods*

<sup>b</sup> Those tumors where the cloning frequency was too low to calculate are reported as <0.01 i.e., <1 cell in 100 with proliferative capacity

<sup>c</sup> ND = not done

<sup>d</sup> (A) represents PTL-P of mechanically derived TIL

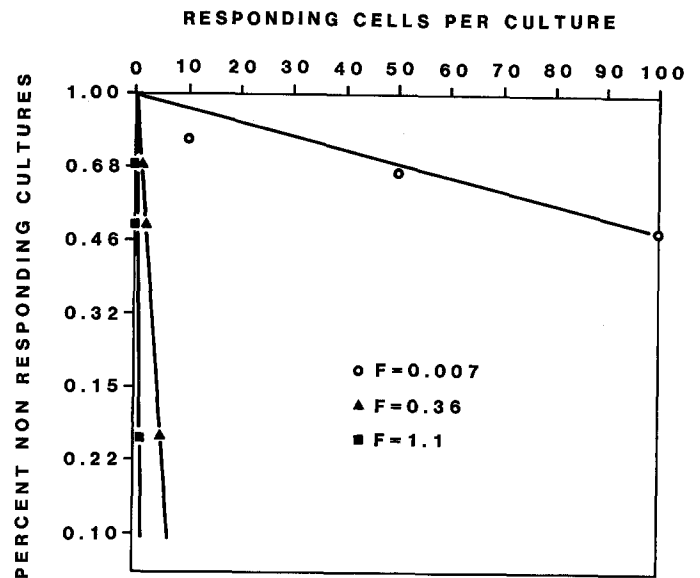
(B) represents PTL-P of enzymatically derived TIL

<sup>e</sup> PTL-P frequencies for normal PBL and tonsil lymphocytes incubated with the same enzyme mixture and under the same conditions used for isolation of TIL

(i.e., 1 in 2 to 1 in 1) in 10 experiments. Also when the patients' PBL were plated according to the number of T11+ cells the PTL-P although higher than that of TIL-T was somewhat lower than that of normal PBL-T (Table 3).

An example of a frequency analysis is shown in Fig. 2 where the TIL-T and PBL-T from patient P. M. were compared with normal PBL-T. It can be seen that the frequency of responding cells was drastically reduced in TIL-T (0.007), compared to the autologous PBL-T (0.36) or to normal PBL-T (1.1)

We considered the possibility that incubations in the presence of enzymes that were necessary for recovery of TIL from solid tumors may interfere with clonogenicity of T lymphocytes in the TIL preparations. For that reason, control PBL and tonsil lymphocytes treated with the same enzyme mixture and incubated under the same conditions as TIL preparations were plated simultaneously with experimental cultures. Proliferating frequencies of control PBL-T and tonsil T lymphocytes were unimpaired even after overnight incubations with the enzymes (Table 3). Also, in one case of breast carcinoma (C. E.), proliferating frequencies of TIL-T in enzymatically prepared and mechanically disaggregated (no digestion with enzymes) preparations were compared. The enzymatically prepared TIL had a better PTL-P frequency than lymphocytes obtained early in the separation procedure from the mechanical suspen-

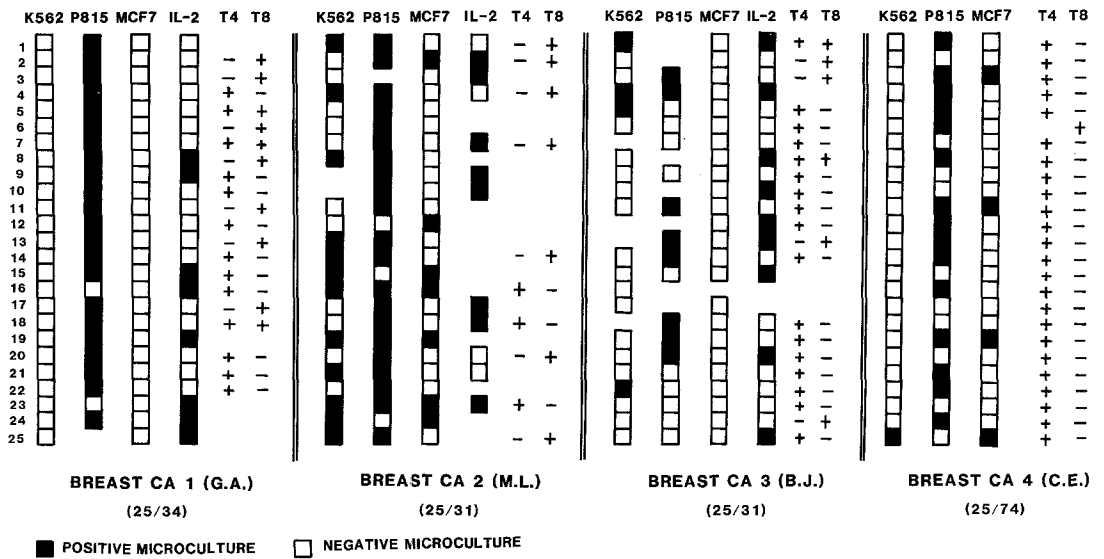


**Fig. 2.** Limiting-dilution analysis of the frequency (F) of proliferating cells in patient P. M.: TIL-T ○—○, autologous PBL-T ▲—▲ and normal PBL-T ■—■. Cells were plated according to the number of T11+ cells at varying cell doses per well in the presence of irradiated feeder cells, 1% PHA (v/v), and an exogenous source of IL-2. After 14–25 days of culture each well was scored microscopically for proliferation. Each point is based on a minimum of 42 microcultures. The regression line was fitted to the data by the minimum  $\chi^2$  method (Taswell)

sions (Table 3). Thus the decreased PTL-P frequency of TIL from human breast carcinomas in our microculture system are a characteristic of these cells and are not due to the experimental conditions used.

#### Clonal analysis of TIL (Fig. 3)

In view of the low PTL-P frequency of TIL, it was necessary to plate them at higher T cell numbers per well to generate microcultures. Indeed, after increasing the number of plated cells to 5, 10, 100, and 200 T11+ lymphocytes per well, proliferating microcultures were obtained. Thus, a total of 170 proliferating microcultures were generated from 4 TIL preparations studied and were expanded for clonal analysis, i.e., cytolytic activities against K562, P815 and an allogeneic breast tumor cell line MCF7 cells. They were also analyzed for IL-2 production and surface phenotype as shown in Fig. 3. Not all of these microcultures could be tested in all the assays listed above, because of differences in their growth: some microcultures did not survive long enough for complete functional evaluation. A total of 116 out of 170 microcultures were phenotyped using double immunofluorescence, only 10 of these contained a mixture of T4+ and T8+ lymphocytes. More than 90% of the microcultures obtained expressed either the T4 (73%) or T8 phenotype (18%) irrespective of determined T4/T8 ratios of TIL suspensions. Operationally, these microcultures were considered as putative clones on the basis of the surface phenotype analysis and because the plating efficiency in three cases did not exceed 3%. In patient C. E., the plating efficiency was 39%, and thus the possibility that some of the microcultures obtained were not monoclonal is much greater. The four breast tumors whose TIL clones were analyzed and phenotyped contained more T8+ than



**Fig. 3.** Clonal analysis of microcultures obtained from four different TIL preparations isolated from breast carcinomas (1–4). In each case, data for 25 representative microcultures are given. Cytotoxicity assays were performed with different target cells (P815 in the presence of a lectin, K562, MCF7) as described in *Materials and methods*. IL-2 synthesis and the expression of T4 and T8 surface antigens on T lymphocytes in each microculture were also determined. Results are represented as ■ = positive or □ = negative. Microcultures which could not be tested are not represented by a square

T4+ lymphocytes in situ (Table 1, the mean T4/T8 ratio =  $0.43 \pm 0.2$ ). The T4/T8 ratio of recovered TIL was determined in only one preparation (no 4, ratio = 3.0) which was cloned and gave 51/53 or 96% of T4+ microcultures. Thus under these culture conditions, proliferating microcultures of TIL from four breast carcinomas were largely of the T4 phenotype irrespective of the predominance of T8+ lymphocytes seen in these tumors by immunohistology.

Functional activities and phenotypes of 25 microcultures representative of the TIL preparations derived from four patients are summarized in Fig. 3. Although patterns of cytolytic activity varied between the groups of microcultures derived from individual TIL preparations, a large number (56%) were able to lyse the P815 target upon lectin activation, indicating that cytolytic T cells were the most frequent cells among growing microcultures. Activity against K562 cells (NK-like function) was variable and usually mediated by cells lytic in the LDCC e.g., in patient 2, 9 clones positive for LDCC also lysed K562 targets. Overall NK activity was demonstrable in 20/56 or 36% of clones in patients 2 and 3. On the other hand, in breast carcinoma no. 1, none of the clones displaying LDCC were positive for NK activity. Indeed of the 34 clones tested none displayed lysis against K562. Also clones from patient 4 were devoid of NK-like activity i.e., of 74 clones tested only 2 were positive. However the frequency of clones with NK activity in the peripheral blood from patient 1 was found to be 0.05, which approximates to the range found in normal PBL (0.07–0.16). Thus the incidence of clones with NK activity among TIL was not always consistent with immunohistology which showed virtually a complete lack of the Leu-7+ and Leu-11+ cells in all 12 breast carcinomas examined. A few were selectively cytotoxic for the allogeneic breast tumor cell line, MCF7.

Strikingly, many of the T4+ microcultures (53%) were cytolytic in LDCC assays (Figs. 3 and 4), and the percentages of specific lysis often approached or even ex-

ceeded those of T8+ microcultures as shown in Fig. 4 for breast carcinomas 1 and 2. The T4+/T8– cytolytic clones were present in all 4 TIL preparations, and in most instances represented the majority of clones displaying a cytolytic potential i.e., 10/22, 3/10, 6/9, and 16/25 clones in patients 1 to 4 respectively were T4+/T8– and positive in the LDCC assay. Very few of these clones displayed NK-like activity. Clones of T lymphocytes derived from normal peripheral blood which express the T4 phenotype are rarely cytolytic for P815 targets in the presence of PHA (Fig. 5). Figure 5 also shows that in comparison to T4+ clones obtained from normal peripheral blood, the T4+ clones derived from breast carcinoma TIL exhibited high specific lysis in LDCC assays.

The IL-2 production (helper function) was also measured in 60 clones (Fig. 3). It can be seen that 8/25, 8/12, and 9/23 clones in patients 1, 2 and 3 respectively were IL-2 producers. Interestingly, several LDCC positive clones, irrespective of their T4+/T8– or T4–/T8+ surface phenotype, were IL-2 producers as well. Thus cytolytic clones able to produce IL-2 were obtained from TIL of three patients with breast carcinoma.

## Discussion

The technology of cloning and clonal analysis has proved to be a powerful tool in analysis of the functional potential of circulating lymphocytes in normal as well as pathological situations [1, 5, 19, 27, 29, 37]. We have used a PHA-dependent microculture system, which allows the clonal expansion of every normal T lymphocyte [27], to investigate the frequency of PTL-P and the incidence of cytolytic T cell precursors in TIL-enriched preparations recovered from human breast carcinomas. These functional data were correlated with a precise in situ analysis of TIL by immunohistology.

T11+ lymphocytes constituted from 30% to 95% of the TIL population recovered and immunohistology per-

LDCC

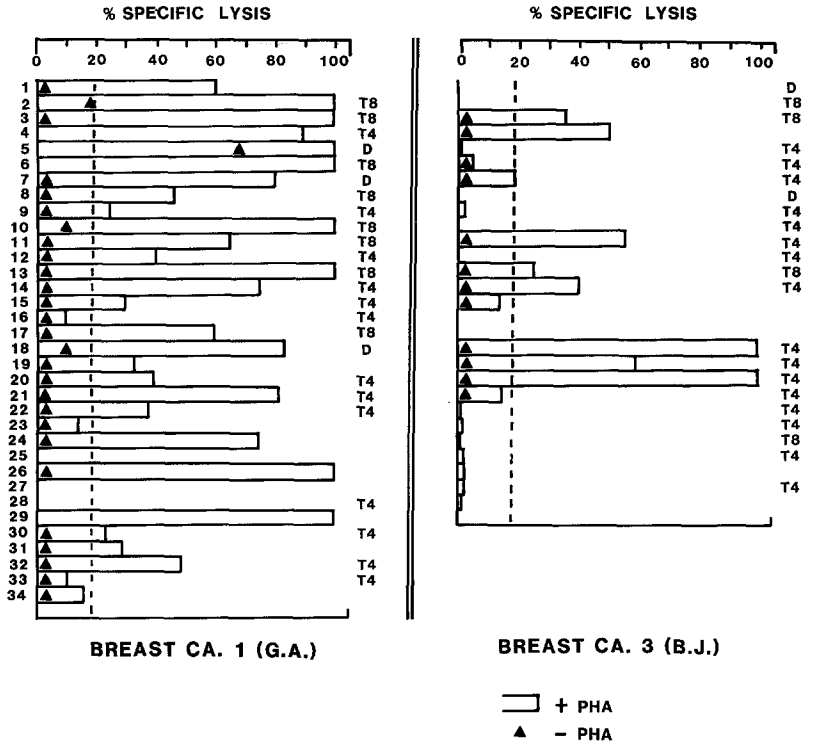


Fig. 4. Cytolytic activities of microcultures in LDCC assays on P815 targets and the expression of T4 and T8 surface antigens on T lymphocytes in each microculture. The TIL from two breast carcinomas (1 and 3) were cloned under limiting-dilution conditions and the expanded microcultures tested for LDCC in the presence and absence of 1% PHA. Note that in several instances T4+/T8--microcultures expressed higher specific lysis than T4-/T8+ microcultures. Dotted lines represent 3 SD over background values given by the spontaneous release of <sup>51</sup>Cr-labeled P815 targets

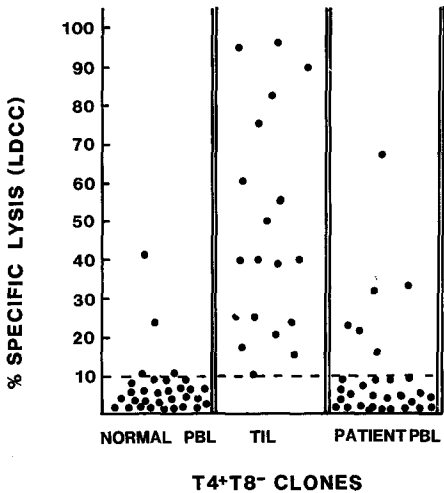


Fig. 5. Comparison of cytolytic activities in LDCC of randomly selected T4+/T8- clones from normal PBL, TIL from breast carcinomas and PBL from patients with breast tumors. The dotted line represents 3 SD over background values given by the spontaneous release of <sup>51</sup>Cr-labeled P815 targets

formed on the breast tumor biopsies from which TIL were extracted confirmed the predominance of T cells in inflammatory infiltrates in the tumor stroma. In addition, immunohistology of tumor sections and immunofluorescence of TIL suspensions showed that only rare T lymphocytes were in the activated state as judged by the expression of IL-2 R. Cytology of TIL on MGG smears was consistent with the histological findings and revealed the presence of small, non blastic lymphocytes.

Quantitative assessment of the proliferating capacity of T cells infiltrating human tumors has not been done previ-

ously in a system that allows under optimal conditions the proliferation of every single resting normal peripheral T cell [27]. In this study, we have shown that in a majority of cases the frequency of PTL-P was greatly reduced. Appropriate control experiments have demonstrated that the low clonogenicity of TIL is a characteristic of these cells and was not due to the experimental conditions used. In a previous report [41], we have shown similarly decreased PTL-P frequencies in TIL from various other tumor types (melanoma, lung carcinoma, colon carcinoma, gliomas), so that these findings are not exclusive for breast tumors.

These low proliferating frequencies are most likely explained by a decreased responsiveness of TIL to PHA. Indeed, we have evidence that human TIL from different tumors, including breast carcinomas, are often poorly responsive to mitogens as populations [23] and similar behavior of TIL has been reported before [38]. Thus, under limiting-dilution conditions, TIL of patients with breast cancer were often poorly clonogenic in the PHA-dependent system. A higher cloning efficiency (15%–42%) obtained in the same system by Ferrini et al. [8] with tumor-associated lymphocytes from ascites of patients with ovarian tumors can perhaps be explained by a different in situ origin of T lymphocytes. In breast tumors, TIL were derived from deep inside the stroma, as evidenced by immunohistology (Fig. 1) of the tumors we studied. Interestingly, the circulating lymphocytes of patients with breast cancer, which show normal PHA responses in bulk cultures [23], had somewhat lower than normal frequencies of PTL-P (Table 3).

It is well documented that the microenvironment of the tumor may be inhibitory for a number of lymphocyte functions [12, 18, 35]. We have shown earlier that incubation of normal PBL with tumor cells leads to the inhibition of lymphocyte functions in populations as well as on a single-



cell level [23]. In fact, the functional behavior of TIL recovered from human tumors is similar to that of normal PBL exposed *in vitro* to tumor cell lines [23].

It is remarkable that in spite of inhibitory influences of the tumor milieu and independently of the frequency of PTL-P, a majority of the growing microcultures from the TIL preparations displayed cytolytic activities (Fig. 3). Whereas the functional profiles of the microcultures obtained in such cases where the PTL-P was  $< 0.01$  can hardly be considered as representative of the total original infiltrate, in two cases (patients C. E. and B. J. with 0.39 and 0.03 PTL-P frequencies, respectively) the high incidence of cytolytic T lymphocyte (CTL) clones (i. e.  $> 60\%$  of the cultures positive in the LDCC) had definite significance regarding the *in situ* situation. Thus, our clonal analysis seems to indicate that CTL precursors (with unknown specificity) are well represented in TIL from breast carcinoma. These results are in agreement with the findings of Ferrini et al. [8] who obtained clones with different cytolytic activities from tumor-associated lymphocytes in human ovarian tumors. In Vose's study [37] there was an indication that in TIL from several human solid tumors, there was an increased frequency of cytotoxic precursors against autologous tumor cells in comparison to PBL. It is likely that selective accumulation of cytolytic cell precursors demonstrated in some animal tumors [4] may also occur in human solid tumors.

Phenotyping of TIL recovered from murine sarcoma virus-induced tumors in mice demonstrated the predominance of Lyt2+ cells [4]. In our hands, the clones obtained from the breast tumor TIL were largely T4 positive (73%). This is not consistent with immunohistology, which showed a greater number of T8+ than T4+ lymphocytes *in situ* in the relevant breast tumors. While this could mean that a selective enrichment in T4+ cells occurred during the TIL isolation procedure, the T4/T8 ratios determined in TIL preparations from various tumors [41] indicated the presence of both T4+ and T8+ lymphocytes in about equal proportions in these preparations. The other possibility, namely, a selective outgrowth of T4+ clones from TIL in our microculture system should be considered. Although such selectivity does not occur with normal PBL-T [26], the low proliferating frequencies of TIL imply a selection in favor of those T lymphocytes among TIL that remain responsive to PHA. Interestingly, even if the cloning efficiency of TIL was high (39% in one of our preparations), the same predominance of T4+ clones (51/53 tested) was observed. Further studies are in progress using highly purified T cells from TIL to clarify this issue.

Our finding that many T4+/T8- clones generated from TIL of breast carcinoma expressed cytolytic activities, is particularly significant. In fact previous reports have already documented the lack of a strict association between phenotype and function in peripheral blood [26]. Also Vose and White [39] reported T4+ autolytic clones generated during mixed lymphocyte tumor culture. More recently Jacobson et al., [15] demonstrated that the majority of measles virus-specific cytotoxic T lymphocytes generated from normal PBL were OKT4+. Interestingly our study also demonstrated an unusually high incidence of T4+ cytolytic clones generated from TIL preparations. As Fig. 4 demonstrates, these T4+ cells lysed P815 targets as efficiently as T8+ clones. In view of the fact that only a very small proportion ( $< 5\%$ ) of T4+ clones from normal

PBL have LDCC activity (see Fig. 5 and [26], it can be concluded that at least some human breast tumors contain frequent T4+ lymphocyte precursors with cytolytic potential. In addition, in many cases, these proliferating T4+/T8- cytolytic cells were also IL-2 producers. Thus T4+/T8- clones had a dual cytolytic and helper-inducer functional potential. Similar findings have been reported by Romagnani et al. in spleens of patients with Hodgkins disease [30]. The reason why TIL from patients with breast carcinomas and perhaps with other malignancies have such a high number of dual cytolytic/helper T cells (an infrequent blood T cell subset) is unclear at present. However it may indicate that these patients mount a cytolytic immune response against unknown antigens perhaps recognized in the context of HLA class II antigens.

Eremin and colleagues [7] reported that lymphocytes infiltrating human primary mammary carcinomas lacked killer and NK cell activities. Depressed NK activity in TIL populations has also been observed in lung tumors [28]; in ovarian tumors [14], and in different human solid tumors [35, 40]. Our clonal analysis of microcultures derived from TIL of breast carcinomas confirms the depletion of NK activity in some breast tumors, but not in others. Even when clones with NK activity were detected, their number was modest in comparison to clones positive for LDCC.

Limited growth of cloned TIL and small sizes of resected breast tumors did not allow a systematic search for cytolytic activity against fresh autologous tumor cells. A few CTL clones (i. e., positive in the LDCC assay) were able to lyse the allogeneic breast carcinoma line MCF7 without mediating NK-like lysis on K562 targets, but the reverse was equally true, i. e., a few clones displaying NK-like lysis and not LDCC activity were also able to lyse the allogeneic tumor target. Also there were CTL clones with lytic activities against both K562 and MCF7 cells, indicating that the antitumor lytic activity of T cells recovered from TIL is extremely heterogeneous.

Our data indicate that T lymphocytes potentially capable of cytolytic functions are present in human breast tumors and that they can be cloned and expanded *in vitro* using TIL populations isolated from these tumors. Further studies of such tumor-derived cytolytic clones are likely to clarify the nature of the interactions between CTL and tumor cells in human solid tumors.

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