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Phenotypic characteristics of tumour-infiltrating lymphocytes in human oesophageal cancer tissues defined by quantitative two-colour analysis with flow-cytometry

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Summary. Phenotypic subpopulations of tumour-infiltrating lymphocytes (TIL) separated from human oesophageal cancer tissues were defined by quantitative two-colour analysis with flow-cytometry (FACScan), and their characteristics were investigated by comparison with peripheral blood lymphocytes (PBL) and intra-oesophageal lymphocytes from noncancerous tissue (IEL) as the controls. Fifteen patients (13 males and 2 females) with squamous cell carcinoma of the oesophagus were entered into the study. Lymphocytes were analyzed by FACScan and the frequencies of the subpopulations were determined using monoclonal antibodies for surface markers. Single colour analysis revealed a predominance of T cells among TIL and a significant reduction of natural killer (NK) cells compared with the controls. Two-colour analysis showed that $CD4^+Leu8^-$ (helper T cells) and $CD8^+CD11b^-$ (cytotoxic T cells) were significantly increased among TIL when compared with the controls. This significant increase of both helper and cytotoxic T cells, which are indispensable components of cellular immunity, strongly implies that TIL are performing a role in the expression of antigen-specific cellular immunity against the tumours. This is the first report of a phenotypic study of human oesophageal cancer that clearly indicates the significance of the TIL and suggests their potential for use as a source of adoptive immunotherapy.

Key words: Oesophageal cancer – Tumour infiltrating lymphocyte – Phenotypic subpopulation – Cell-mediated immunity – Flow-cytometry

Tumour-infiltrating lymphocytes (TIL) have been suggested as expressions of a tumour-specific host response in various solid tumours including oesophageal cancer, judging from their correlations with the length of survival after surgery (Moore and Foote 1949; Takahashi 1961; Martin and Bechwith 1968; Bennett et al. 1971; Berg 1971; Black et al. 1972; Shiozaki et al. 1983). Experimental evidence has supported this hypothesis and implies a tight correlation between TIL and cell-mediated immunity (Kikuchi et al. 1972; Shimokawa et al. 1982; Vose 1982; Hiratsuka et al. 1984).

It has also recently been reported that TIL cultured with interleukin-2 (IL-2) have much more tumour-specific cytotoxicity than lymphokine-activated killer (LAK) cells both in vitro and in vivo (Rosenberg et al. 1986; Rabinowich et al. 1987; Kuppner et al. 1988; Takagi et al. 1989). Thus, TIL have been seen as possible specific effectors for autologous tumour cells and as an important source for adoptive immunotherapy. However, it still remains unclear whether or not the TIL in human oesophageal cancer are really the effector cell population of a specific immune reaction recognizing tumour cells as antigens in vivo.

In order to elucidate their functions, we applied flow-cytometric quantitative two-colour analysis to TIL separated from surgically-resected oesophageal cancer sepcimens. Double staining of the surface markers relevant to function was performed and the phenotypic make-up was compared with that of peripheral blood lymphocytes (PBL) and intra-oesophageal lymphocytes from non-cancerous tissue (IEL) obtained from the same patients.

Materials and methods

Fifteen patients with squamous cell carcinoma of the oesophagus, who underwent subtotal oesophageal resection, were examined in this study. Each patient gave informed voluntary consent. None of the patients had received any therapy prior to

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surgery. The patients ranged in age from 44 to 76 years (mean age = 56) and the male to female ratio was 13:2.

The histological features of the specimens for flow-cytometric analysis were assessed using H &E stained sections taken from immediately adjacent to the specimens used for flow-cytometry. The terminology used in this report for location, staging, and histological type is derived from the Tumour-Node-Metastasis staging system (UICC 1987).

Samples of 15 ml of heparinized venous blood were obtained from all patients on the morning of surgery before the administration of any narcotic agent, and the mononuclear cells were separated by standard Ficoll-Hypaque gradient centrifugation (Boeyum 1968) using LSM (Organon Teknika Corporation, North Carolina, USA) as the separating medium. In brief, blood samples were diluted twice to half their original concentration with Hanks' balanced salt solution (HBSS; Biken, Japan), floated onto LSM, and centrifuged at $400 \times g$ for 30 min. The mononuclear cells were collected at the gradient interface, washed twice with HBSS, and resuspended in HBSS containing 0.1% bovine serum albumin (BSA) and 0.02% NaN_3 (medium A) at a concentration of 5.0×10^6 cells/ml. Cell viability was confirmed to be greater than 98% by trypan blue dye exclusion.

For the isolation of TIL and IEL rich aliquots, tumour tissue and non-cancerous oesophageal tissue samples were obtained from the surgically-resected specimens. Processing was initiated within 2 h of resection. The weights of the specimens used to obtain TIL and IEL ranged from 1 to 2.6 g and 2 to 4 g, respectively. Necrotic tissue and connective tissue was removed. Then the specimen was minced with scissors and suspended in RPMI 1640 (Biken, Japan) containing collagenase (type IV, 200 units/ml, Sigma Chemical Co., St. Louis, MO, USA) and 10% fetal bovine serum (FBS). The suspension was stirred for 2 h at 37° C, passed through a fine nylon mesh filter, harvested by centrifugation, washed twice with HBSS, and suspended in HBSS containing 10% FBS. The cell suspension was then floated on LSM and centrifuged at $400 \times g$ for 20 min. Cells at the interface consisted of lymphocytes, monocytes, macrophages, and tumour cells. They were collected and adjusted to concentrations of from 5×10^5 to 5×10^6 cells/ml in medium A. The viability of the TIL and IEL was confirmed to be greater than 98% by trypan blue dye exclusion, and the numbers of TIL and IEL per weight of wet tissue sample were calculated.

Table 1 shows the monoclonal antibody combinations which were used in this study and also shows the phenotypes they defined. All of them were conjugated with either fluorescein (FITC) or phycoerythrin (PE) (Beckton Dickinson Monoclonal Antibody Center, Mountain View, CA). Leu7 (Si and Whiteside 1983; Lanier et al. 1983), Leu8 (Gatenby et al. 1982; Kansas et al. 1985), and Leu15 (Landay et al. 1983) are the monoclonal antibodies which can detect most of the specific functional capabilities of lymphocytes when paired with the major appropriate antibodies. The PBL, TIL, and IEL (1×10^5 cells each) were incubated with 10 µl of the combined antibodies shown in Table I for 20 min at 4° C. The cells were then washed once with HBSS and resuspended in a further 0.4 ml of HBSS. Two-colour analysis of the immunofluorescent staining was performed using a flow-cytometer (FACScan; Beckton Dickinson, Mountain View, CA, USA) according to the method stated below.

Fluorescence-conjugated antibody staining was detected using a FACScan for flow-cytometric analysis. Signal acquisition was triggered on forward light scatter and there was simultaneous acquisition of forward and side light scatter (FSC and SSC) and of optimally compensated two-colour log-fluorescence signals. Data were acquired by a computer module (Hewlett-Packard, series 9000, type 310, USA) using data acquisition software (Consort 30, Beckton Dickinson), and were stored on 3.5-inch floppy disks. For each experiment, a minimum of 10000 and a maximum of 30000 cells was counted.

Table 1. Monoclonal antibody combinations and the phenotypes they defined

FITC conjugated	PE conjugated	Defined phenotypes
Leu4 (CD3)		pan T cell
	Leu12 (CD19)	pan B cell
Leu2a (CD8)		cytotoxic/ suppressor T cell
	Leu3a (CD4)	helper/inducer T cell
Leu3a (CD4)	Leu8	$CD4^+Leu8^+$: inducer T cell $CD4^+Leu8^-$: helper T cell
Leu2a (CD8)	Leu15 (CD11b)	$CD8^+CD11b^+$: suppressor T cell $CD8^+CD11b^-$: cytotoxic T cell
Leu7 (HNK-1)	Leu11c (CD16)	$CD16^+$: Natural Killer $CD16^+Leu7^-$: most potent NK
HLe-1 (CD45)		$CD45^+$: pan leukocyte

FITC = Fluorescein, PE = Phycoerythrin, CD = Cluster differentiation. Leu series monoclonal antibodies were purchased from Becton-Dickinson. Mountain View, CA, USA

Acquired data files were analyzed by the program for analysis in the Consort 30 software which simultaneously determined two-colour fluorescence data on samples gated by FSC and SSC. For PBL analysis, stored data from the floppy disks were gated through the window established for lymphocytes by their FSC and SSC characteristics (Fig. 1A). For TIL and IEL analysis, in order to separate lymphocytes from contaminating cells such as monocytes, macrophages, and viable tumour cells, the same window was used as for PBL (Fig. 1B), and more than 2000 cells were gated from each specimen. The two-colour fluorescence data of the gated cells were indicated by contour lines and dots on a figure divided into four contiguous areas which were designated as "quadrants" (Fig. 2), and the percentage of cells in each quadrant was calculated simultaneously.

The frequency of lymphocytes among the gated cells was confirmed by the percentage of $CD45^+$ cells present. If the gated population showed more than 5% contamination, the frequency of each phenotypic subpopulation was compensated according to the percentage of $CD45^+$ cells.

The data were analyzed for statistical significance by the non-parametric Wilcoxon test, and the result was considered significant if the P value was less than 0.05.

Results

All of the primary tumours were located in the intrathoracic oesophagus. Tumour size ranged

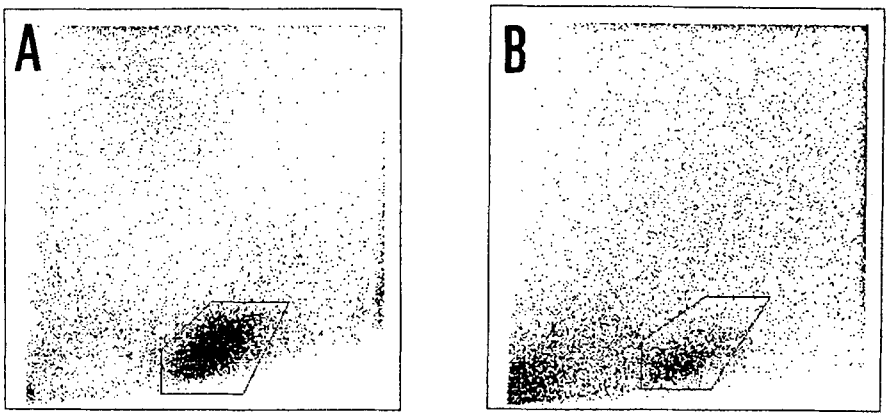

Fig. 1. Gated windows for lymphocytes on FSC and SSC characteristics. The window established for lymphocytes was used for PBL (**A**). The same window was used for TIL (**B**) and IEL (not shown). FITC conjugated HLe-1 (CD45, pan leukocyte) monoclonal antibody was used as the positive control. *FSC* = forward light scatter, *SSC* = side light scatter

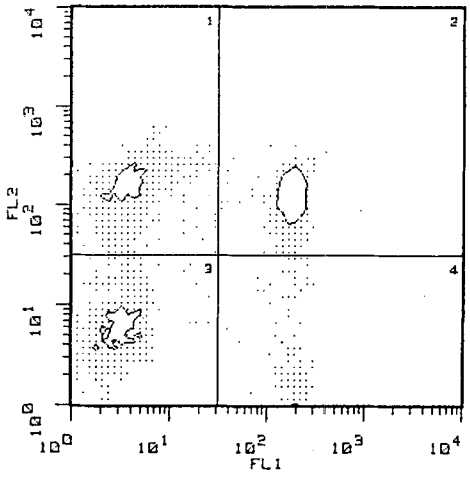

Fig. 2. Contour line graph of two-colour analysis. The dots and the contour lines indicate the numbers of cells determined by dual-fluorescence within the gated populations. This is a graph of the analysis of $CD4^+$ and $Leu8^+$ cells in case 7. FL 1 indicates green fluorescence (*CD4-FITC*), and FL2 indicates orange-red fluorescence (*Leu8-PE*). Four contiguous areas, which were designated as "quadrants", were defined by the marked lines. The percentage of cells in each quadrant was calculated simultaneously. *dot* = single cell, —— = 10 cells

from 2.6 to 8.3 cm in maximum diameter. Most of the tumours (11/15) were classified as T3, 2 were T2, and 2 were T4. Twelve of the 15 subjects had regional lymph node metastases (N1) and the other 3 had no lymph node metastases (N0). Distant metastasis was found intraoperatively in one case. From these findings, 3 of the cases were classified into stage IIA, 1 into stage IIB, 10 into stage III, and 1 into stage IV. Histological examination revealed that all of the tumours were squamous cell carcinoma and they showed a normal distribution of differentiation (well differentiated: 3, moderately differentiated: 8, poorly differentiated: 4).

The number of TIL per gram of wet tissue was $(2.2 \pm 1.8) \times 10^6$ (mean $\pm$ SD), which was significantly increased compared with the number of IEL ($(2.4 \pm 2.4) \times 10^5$; $P < 0.005$).

The means and standard deviations of the frequencies of the subpopulations of TIL, PBL, and IEL detected by the major antibodies are shown in Fig. 3. Anti-CD3 and anti-CD19 antibodies were used to determine the T/B ratio in the 3 populations. The frequency of $CD3^+$ cells in the TIL

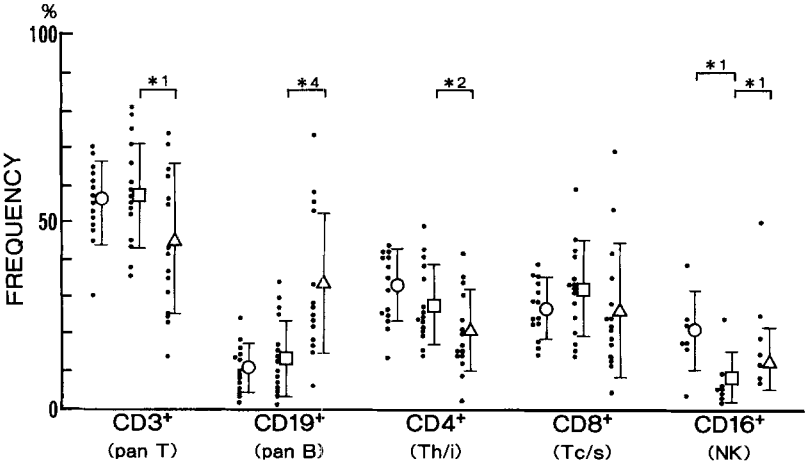

Fig. 3. Phenotyping of the TIL, PBL, and IEL populations as defined by the major antibodies. *Symbols* and *vertical bars* represent the mean $\pm$ SD, and a *dot* indicates the frequency of each specimen. Single-colour analysis of the TIL population revealed a predominance of T cells and a significant reduction of the frequency of NK cells compared with the controls. o = PBL, □ = TIL, △ = IEL. *Th/i* = helper/inducer T cell, *Tc/s* = cytotoxic/suppressor T cell, *NK* = Natural Killer cells. Significance of difference; * = $P < 0.05$, ** = $P < 0.025$, *** = $P < 0.01$, **** = $P < 0.005$

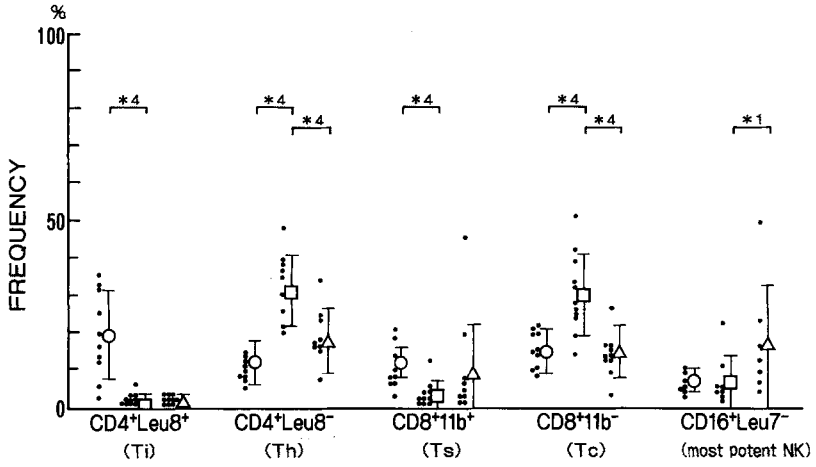

Fig. 4. Phenotypes of TIL, PBL, and IEL populations defined by two-colour analysis (mean ± SD). Two-colour analysis clearly revealed a significant increase of the helper and cytotoxic T cell subpopulations. ○ = PBL, □ = TIL, △ = IEL. Significance of difference; * = $P<0.05$, **** = $P<0.005$

population was 58.2 ± 14.1% (mean ± SD), which was similar to that for PBL (55.3 ± 10.8%), but higher than that for IEL (45.6 ± 19.8%; $P<0.05$). In contrast, $CD19^+$ cells comprised 14.0 ± 10.5% of the TIL population, which was similar to the proportion in PBL (10.9 ± 6.5%), but was much lower than in the IEL population (33.8 ± 18.9%, $P<0.005$). The T/B ratio of TIL (28.6 ± 84.7) was hence significantly higher than that of IEL (2.0 ± 1.4, $P<0.005$) but was similar to that for PBL.

Anti-CD4 and anti-CD8 antibodies were used to determine major T cell subsets such as helper/inducer T cells (Th/i) and cytotoxic/suppressor T cells (Tc/s). The $CD8^+$ cells had a similar frequency of occurrence in all 3 cell populations (TIL: 31.8 ± 13.1%; PBL: 27.3 ± 7.7% and IEL: 27.2 ± 17.6%). In contrast, $CD4^+$ cells were found at a similar frequency among TIL (28.2 ± 10.6%) and PBL (32.8 ± 10.1%), but were significantly less common among IEL (10.9 ± 10.8%).

The frequency of $CD16^+$ lymphocytes among TIL was only 8.2 ± 7.4%, which was significantly lower than for both PBL and IEL (PBL: 20.5 ± 11.3%, $P<0.05$, IEL: 13.5 ± 7.6%, $P<0.05$).

The $CD4^+$, $CD8^+$, and $CD16^+$ cells of 10 patients (cases No. 6 to 15) were examined in detail by analyzing the coexpression of their second antibodies (Leu8, CD11b, and Leu7, respectively; Table 1, Fig. 4).

The average frequency of $Leu8^+$ cells among the $CD4^+$ cells in the TIL population was only 5.0 ± 4.3%, thus the frequency of $CD4^+Leu8^+$ cells was 2.0 ± 2.3% and that of $CD4^+Leu8^-$ cells was 31.5 ± 9.9%. Thus, the percentage of $CD4^+Leu8^-$ cells in the TIL population was significantly higher than in both the PBL and IEL populations (PBL: 12.1 ± 6.3%, $P<0.005$; IEL: 17.5 ± 8.8%, $P<0.005$), while the frequency of $CD8^+Leu8^+$ cells was much lower than in the PBL population (19.1 ± 11.8, $P<0.005$).

The average frequency of CD11b+ cells among CD8+ TIL was 9.7 ± 8.0%, so the frequency of $CD8^+CD11b^+$ cells was 3.2 ± 3.7% which was significantly lower than in the PBL population (12.2 ± 7.4%, $P<0.005$). The frequency of $CD8^+CD11b^-$ cells in the TIL population was 29.6 ± 11.3%, which was significantly higher than those in both the PBL and IEL populations (PBL: 15.4 ± 6.3, $P<0.005$; IEL: 14.6 ± 6.6, $P<0.005$).

Cells which are $CD16^+Leu7^-$ are considered to be the most potent NK cells. The frequency of $CD16^+Leu7^-$ cells in the TIL population was 6.7 ± 7.8%, which was similar to that in the PBL population (6.6 ± 2.9%), and was significantly lower than that in the IEL population (17.1 ± 15.8%, $P<0.05$).

Discussion

We attempted to define the functions of TIL in human oesophageal cancer by their phenotypic characteristics. The present study is unique in two ways compared with previous reports on the phenotyping of TIL (Shimokawa et al. 1982; Hiratsuka et al. 1984; Goettlinger et al. 1985; Hurlimann and Saraga 1985). One is the application of two-colour analysis with flow-cytometry, and the other is the

comparison made using IEL and PBL as controls in the same patients. The use of two-colour analysis with flow-cytometry was designed to bring us quantitative and detailed information about the phenotypes in the TIL population. The comparison with controls was performed to clarify shifts of the phenotypic make-up of the TIL from the original phenotypes which might be closer to the PBL and/or IEL phenotypes.

We were clearly able to show the characteristics of the phenotypes in the TIL population. With single-colour analysis, we showed a predominance of T cells and a significant reduction of the frequency of natural killer (NK) cells among TIL compared with those in the controls. With two-colour analysis using the appropriate pairs of antibodies, we also showed that the frequencies of $CD4^+Leu8^-$ (helper T cells; Th) and $CD8^+CD11b^-$ cells (cytotoxic T cells; Tc) in the TIL population were significantly greater than in the control populations. These results strongly suggest that T cells have a greater role in TIL functioning than do NK cells, and that Th and Tc might play a leading role in TIL activities. Since both Th and Tc are indispensable components of cellular immunity (Cantor and Boyse 1977; Cantor and Gershon 1979), these results indicate that TIL in human oesophageal cancer may be an expression of antigen-specific cellular immunity against the tumours. The potential of TIL for use in adoptive immunotherapy is also suggested. The increased numbers of TIL per weight of wet tissue compared to IEL also support this proposition.

We have shown phenotypic evidence for the first time in human oesophageal cancer that TIL might be expressing a specific anti-tumour response of the cancer-bearing host and might have a potential value for use in adoptive immunotherapy. The controversy caused by previous studies using the immunohistochemical staining of surface markers may be clarified to some degree by the present study.

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References

Bennett SH, Futrell JW, Roth JA, Hoye RC, Ketcham AS (1971) Prognostic significance of histologic host response in cancer of the larynx or hypolarynx. Cancer 28:1255–1256

Berg JW (1971) Morphological evidence for immune response to breast cancer. A histological view. Cancer 28:1453–1456

Black MM, Freeman C, Mork T, Harvei S, Custer SJ (1972) Prognostic significance of microscopic structure of gastric carcinomas and their regional lymph nodes. Cancer 27:703–711

Boeyum A (1968) Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest 21 [Suppl 97]:77–89

Cantor H, Boyse EA (1977) Regulation of cellular and humoral immune responses by T-cell subclasses. Cold Spring Harbor Symp Quant Biol 41:23–32

Cantor H, Gershon RK (1979) Immunological circuits: cellular composition. Fed Proc 38:2058–2064

Gatenby PA, Kansas GS, Xian CY, Evans RL, Eagleman EG (1982) Dissection of immunoregulatory subpopulations of T-lymphocytes within the helper and suppressor sublineages in man. J Immunol 129:1997–2000

Goettlinger HG, Rieber P, Gokel JM, Lohe KJ, Riethmueller G (1985) Infiltrating mononuclear cells in human breast carcinoma: predominance of T4+ monocytic cells in the tumor stroma. Int J Cancer 35:199–205

Hiratsuka H, Imamura M, Ishii Y, Kohama G, Kikuchi K (1984) Immunohistologic detection of lymphocyte subpopulations infiltrating in human oral cancer with special reference to its clinical significance. Cancer 53:2456–2466

Hurlimann J, Saraga P (1985) Mononuclear cells infiltrating human mammary carcinomas: immunohistochemical analysis with monoclonal antibodies. Int J Cancer 35:753–762

Kansas GS, Wood GS, Eagleman EG (1985) Maturational and functional diversity of human B-lymphocytes delineated with anti-Leu 8. J Immunol 134:3003–3006

Kikuchi K, Kikuchi Y, Phillips ME, Southam CM (1972) Tumor specific, cell-mediated immune resistance to autochthonous tumors. Cancer Res 32:516–521

Kuppner MC, Hanou MF, Tribolet N (1988) Immunohistological and functional analysis of lymphoid infiltrates in human glioblastomas. Cancer Res 48:6926–6932

Landay A, Gartland GL, Clement LT (1983) Characterization of a phenotypically distinct subpopulation of Leu-2a+ cells that suppresses T-cell proliferative responses. J Immunol 131:2757–2762

Lanier LL, Le AM, Phillips JH, Warner NL, Babcock GF (1983) Subpopulations of human natural killer cells defined by expression of the Leu-7(HNK-1) and Leu-11(NK-15) antigens. J Immunol 131:1789–1796

Martin RF, Bechwith JB (1968) Lymphoid infiltrates in neuroblastomas: their occurrence and prognostic significance. J Pediatr Surg 3:161–164

Moore OS Jr, Foote FW Jr (1949) The relatively favorable prognosis of medullary carcinoma of the breast. Cancer 2:635–642

Rabinowich H, Cohen R, Bruderman I, Steiner Z, Klajman A (1987) Functional analysis of mononuclear cells infiltrating into tumors: lysis of autologous human tumor cells by cultured infiltrating lymphocytes. Cancer Res 47:173–177

Rosenberg SA, Spiess P, Lefreniere R (1986) A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. Science 233:1318–1321

Shimokawa I, Imamura M, Yamanaka N, Ishii Y, Kikuchi K (1982) Identification of lymphocyte subpopulations in human breast cancer tissue and significance: an immunoperoxidase study with anti-human T- and B-cell sera. Cancer 49:1456–1464

Shiozaki H, Mizunoya S, Okagawa K, Ueno K, Han S, Kobayashi K, Kosaki G (1983) Significance of the lymphoid stroma at the infiltrative margin of esophageal cancer (in Japanese). Jpn J Gastroent Surg 16:1615–1621

Si L, Whiteside TL (1983) Tissue distribution of human NK cells studied with anti-Leu-7 monoclonal antibody. J Immunol 130:2149–2156

Takagi S, Chen K, Schwarz R, Iwatsuki S, Herberman RB, Whiteside TL (1989) Functional and phenotypic analysis of tumor-infiltrating lymphocytes isolated from human primary and metastatic liver tumors and cultured in recombinant interleukin-2. Cancer 63:102–111

Takahashi K (1961) Squamous cell carcinoma of the esophagus: stromal inflammatory cell infiltration as a prognostic factor. Cancer 14:921–933

UICC (International Union Against Cancer) (1987) TNM classification of malignant tumors, 4th edn. Springer, Berlin Heidelberg New York

Vose BM (1982) Quantitation of proliferative and cytotoxic precursor cells directed against human tumors: limiting dilution analysis in peripheral blood and the tumor site. Int J Cancer 30:135–142

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