Virchows Archiv A Pathol Anat (1990) 417:311–317

Virchows Archiv A
Pathological Anatomy and Histopathology

Principal lymphocyte subpopulation in local host response to human oesophageal cancer

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Received March 10, 1990 / Accepted May 21, 1990

Summary. We investigated what subpopulations of tumour-infiltrating lymphocytes (TIL) play a key role in in vivo function and what determines the degree of local host response represented by lymphocyte infiltration in human oesophageal cancer. We examined the increased subpopulation of TIL in "good responders" (GR) (patients with intensively TIL infiltrated tumours) when compared with "poor responders" (PR) (patients with weakly TIL infiltrated tumours). The frequency of each subpopulation was determined by quantitative flow-cytometric measurement on TIL separated from fresh tumours. Of TIL in GR, the frequency of $CD3^+$ cells increased significantly ($P<0.05$) but the frequencies of $CD16^+$, $Leu7^-$, and $CD16^+$ $Leu7^-$ cells were low and did not increase significantly compared with those in PR. With respect to T-cell subsets of TIL in GR, the frequency of $CD8^+$ cells was significantly higher than that in PR ($P<0.01$), and $CD4^+/CD8^+$ ratio was lower than that in PR ($P<0.025$). On two-colour analyses, most of $CD8^+$ cells (cytotoxic/suppressor T-cells: Tc/s) did not co-express CD11b and the frequency of $CD8^+CD11b^-$ cells (cytotoxic T-cell: Tc) increased significantly compared with that in PR. Clinicopathological and phenotypic analysis of peripheral blood lymphocytes revealed that there are no major differences in general immunocompetence between GR and PR. These results suggest that Tc/s, especially Tc, might play a key role in local host response. They also suggest that not only the general immune status of the host but also the identification of class I major histocompatibility complex antigens by the host at the tumour site may strongly affect the degree of host response in oesophageal cancer.

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Key words: Oesophageal cancer – Tumour-infiltrating lymphocytes – Lymphocyte subpopulation – Flow cytometry

Introduction

The degree of lymphocyte infiltration into the tumour has been reported to have a positive correlation with prognosis in oesophageal cancer (Takahashi 1961; Shiozaki et al. 1983, 1987) as well as in other types. From these clinicopathological observations, tumour-infiltrating lymphocytes (TIL) are suggested to be the effector cells of a potentially protective immune host response against tumour cells in vivo. Recent reports have showed that TIL expanded with interleukin-2 (IL-2) have enhanced in vitro cytotoxicity against tumour cells compared with peripheral blood lymphocytes (PBL) expanded in the same way (Rosenberg et al. 1986; Rabinowich et al. 1987; Takagi 1989). Adoptive immunotherapy utilizing TIL (AIT-TIL) is considered to be promising in vivo (Rosenberg et al. 1986; Topalian et al. 1988).

It is suggested that these functions of human TIL are exerted by T-lymphocytes. Animals which have been immunized against a tumour can specifically reject subsequent challenges by tumour cells with infiltration of mononuclear cells, composed mainly of T-lymphocytes (Kikuchi et al. 1972; Ishii 1984). However, it is still unclear which T-cell subpopulations of human TIL actually play a key role in their in vivo function and what determines the degree of local host response which is represented by the lymphocyte infiltration into the tumour.

Analysis of lymphocyte phenotypes can be helpful, since they primarily have correlations with functions and

restricted antigens such as class I and II major histocompatibility complex (MHC) gene products (Kansas et al. 1985). We have recently reported a flow-cytometric study in which TIL in human oesophageal cancer tissues were found to consist mainly of T-lymphocytes with increased frequencies of both $CD8^+CD11b^-$ and $CD4^+Leu8^-$ cells when compared with those of PBL and lymphocytes in normal oesophageal tissues (Tahara et al. 1990). Our study implies the existence of a specific anti-tumour immune response which is exerted by these lymphocyte subpopulations. In order to make more detailed and direct analysis on TIL using flow cytometry, we have examined the increasing subpopulation of TIL in "good responders" (GR), patients with intensively TIL infiltrated tumours, because of their better prognosis when compared with "poor responders" (PR). We also measured the phenotypic characteristics of PBL, which are commonly used for immune surveillance of patients (Reinherz et al. 1978, 1980; Carney et al. 1981), in order to determine the correlation between the general immunocompetence and local host response.

Patients and methods

Nineteen patients with squamous cell carcinoma of oesophagus entered the present study with voluntary, informed consent. None of them had received any therapy prior to the surgery. The patients ranged in age from 44 to 76 years (mean age = 58.1) and the male to female ratio was 8.5:1.

The histological features of the specimens were evaluated by two trained pathologists using haematoxylin and eosin (H & E) stained sections taken from the tissue adjacent to the specimens used for flow cytometry. The terminology used in this report is derived from the tumour-node-metastasis staging system (UICC 1987). Degrees of local host response to tumour cells were determined by the histological degree of lymphocyte infiltration according to the standards reported previously by Shiozaki et al. (1983). In brief, they were discriminated into four grades by their average counts at high power view (×400) in five fields of the infiltrative margin of the oesophageal cancer; scanty (±, less than 10), slight (+, 10–100), moderate (++, 100–200) and marked (+++, more than 200) (Fig. 1). The cases with "scanty" and "slight" lymphocyte infiltration were defined as PR, and ones with "moderate" and "marked" as GR.

TIL-rich samples and PBL were isolated by the method which we have reported previously (Tahara et al. 1990). The number and viability of the TIL was measured and confirmed to be greater than 98% by trypan blue dye exclusion, and numbers of TIL per gram of wet tissue weight (TIL-No) were calculated. Processing was initiated within 2 h of resection.

The numbers and subpopulations of PBL in the same patients were also measured in this study. Heparinized venous blood samples were obtained prior to surgery and the number of PBL were measured by routine laboratory examination. PBL for phenotypic studies were separated by standard Ficoll-Hypaque gradient centrifugation (Boeyum 1968) using LSM (Organon Teknika Corporation, North Carolina, USA) as the separating medium. Cell viability was confirmed to be greater than 98% by trypan blue exclusion.

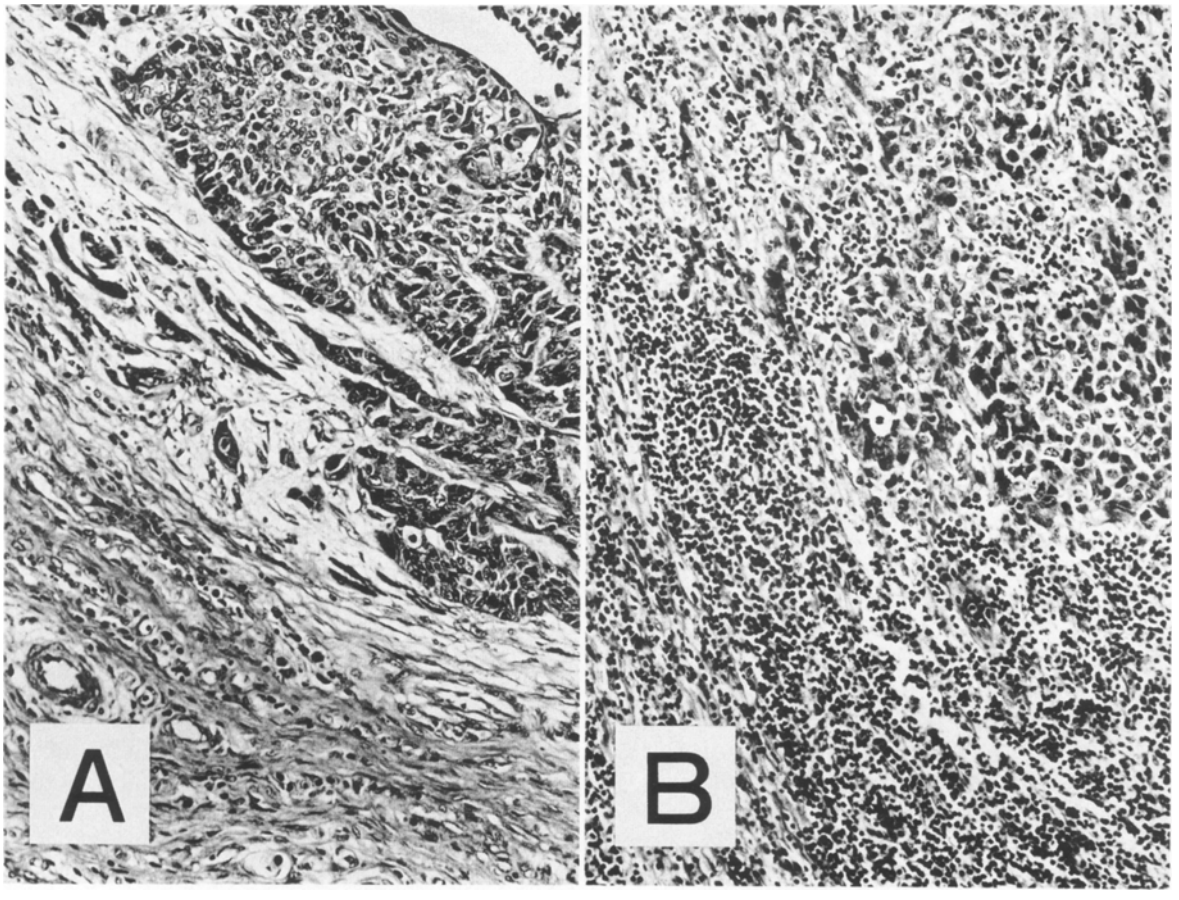

Fig. 1A, B. Determination of the degree of local host response. Degrees of local host response to oesophageal cancer cells were determined by the degrees of lymphocyte-infiltration using haematoxylin and eosin (H & E) stained sections. **A** (×50) and **B** (×50) are typical figures of scanty and marked lymphocyte infiltration in the margin of primary tumour

Table 1. Clinical and histological findings of subjects

Host response	Case no.	Age (years)	Sex	Location[a]	TNM Staging			Stage	Histological type[b]	Lymphocyte infiltration
					T	N	M			
Poor	1	56	M	lower	3	1	1	IV	Mod.	±
	2	56	M	lower	3	0	0	IIA	Poor	±
	3	68	F	lower	2	1	0	IIB	Poor	±
	4	59	M	upper	3	1	0	III	Mod.	±
	5	62	M	mid	3	1	0	III	Poor	±
	6	51	M	lower	3	1	0	III	Mod.	+
	7	53	M	mid	3	1	0	III	Mod.	+
	8	60	M	mid	3	1	0	III	Mod.	+
	9	54	M	mid	3	1	0	III	Mod.	+
	10	57	M	mid	3	1	0	III	Well	+
	11	64	M	lower	3	1	0	III	Poor	+
Good	1	61	M	mid	4	1	0	III	Well	++
	2	54	M	lower	3	1	0	III	Mod.	++
	3	69	M	mid	4	1	0	III	Mod.	++
	4	60	M	mid	3	1	0	III	Well	++
	5	44	M	upper	2	0	0	IIA	Well	+++
	6	56	M	lower	3	1	0	III	Poor	+++
	7	76	F	lower	3	0	0	IIA	Poor	+++
	8	44	M	mid	4	1	0	III	Well	+++

[a] Location of primary tumour in oesophagus: upper = upper thoracic portion, mid = mid-thoracic portion; lower = lower oesophagus
[b] Histological type of squamous cell carcinoma: well = well-differentiated; mod. = moderately differentiated; poor = poorly differentiated

Immunofluorescent labelling and two-colour analysis of isolated lymphocytes was performed in the manner shown in our previous report (Tahara et al. 1990) by a flow cytometer (FACScan; Beckton Dickinson, Mountain View, Calif., USA).

Fluorescence-conjugated antibody staining was detected using a FACScan (a minimum of 10000 and a maximum of 30000 cells was counted in each experiment) and the data acquired were analysed by software (Consort 30) by the method reported previously (Tahara et al. 1990). The frequency of each subset in TIL and PBL was measured as a percentage of the frequency among the optimally gated cells. Absolute numbers of each subset per gram of tumour weight were calculated as a product of TIL-No and frequency.

Statistical significance was examined with the non-parametric Wilcoxon test. A result was considered significant if the P value was less than 0.05.

Results

The clinico-pathological findings in the subjects are shown in Table 1 divided into two groups by the histological responses of the host (PR and GR).

PR (11 patients) ranged in age from 51 to 68 years [mean age $\pm$ standard deviation (SD) $= 58.2 \pm 5.1$] and the male to female ratio was 10:1. In GR (8 patients) ranged in age from 44 to 76 years (58.0 ± 11.1) and the male to female ratio was 7:1. There was no significant difference between the two groups with respect to the age and sex using the χ^2 test.

All of the primary tumours were located in the thoracic oesophagus. The TNM stages in each group mainly consisted of stage III (8/11 in PR and 6/8 in GR). There was no significant difference in tumour stage between the two groups.

The ratio of moderately differentiated squamous cell carcinoma in PR (6/11) was higher than in GR (2/8) but there was no significant difference between the two groups.

The weights of the specimens for TIL ranged from 1 to 2.6 g, and the number of TIL in GR per gram of wet tissue was $(3.39 \pm 1.36) \times 10^6$, which was significantly greater ($P < 0.005$) than that in PR ($(8.25 \pm 6.76) \times 10^5$/g) (Fig. 2).

We measured the frequencies of $CD3^+$, $CD19^+$, $CD16^+$, and $Leu7^+$ cells to determine the frequencies

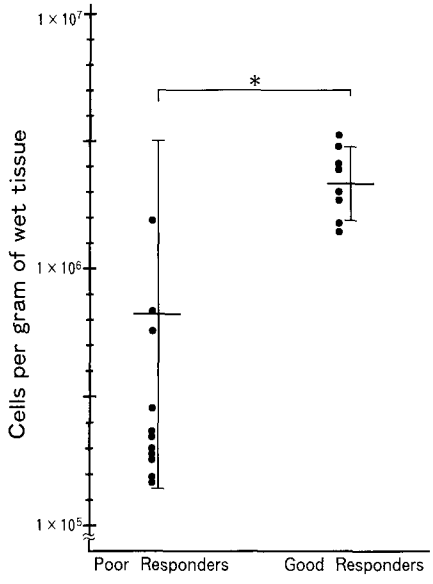

Fig. 2. Numbers of tumour-infiltrating lymphocytes (TIL) per gram of wet tissue in poor responders (PR) and good responders (GR). Mean TIL numbers per gram in GR were significantly higher than those in PR ($P < 0.01$)

of T-, B- and NK-cells (Fig. 3). The frequency of $CD3^+$ cells (pan T-cells) of TIL in GR ($65.9 \pm 9.5\%$) was significantly higher than that in PR ($55.0 \pm 14.3\%$; $P < 0.05$); however the frequency of $CD19^+$ cells (pan B-cell) of TIL in GR ($10.9 \pm 8.0\%$) showed no significant difference compared with those in PR ($17.6 \pm 10.8\%$). The frequency of $CD16^+$ cells and $Leu7^+$ cells (NK-cells) of TIL in GR ($6.0 \pm 2.2\%$, $14.8 \pm 7.3\%$) was low, and had no significant difference compared with those of TIL in PR ($9.7 \pm 8.2\%$, $14.2 \pm 16.3\%$).

Major subpopulations of TIL T-cells, such as $CD4^+$ and $CD8^+$ cells, were examined for each group and the groups compared (Fig. 3). The frequency of TIL $CD4^+$ cells (helper/inducer T-cells: Th/i) in GR ($27.0 \pm 10.6\%$) was slightly lower than that in PR ($31.9 \pm 9.5\%$) but there was no significant difference. The frequency of TIL $CD8^+$ cells (cytotoxic/suppressor T-cells: Tc/s) in GR ($39.3 \pm 11.8\%$) was significantly higher than that in PR ($25.8 \pm 8.1\%$; $P < 0.01$).

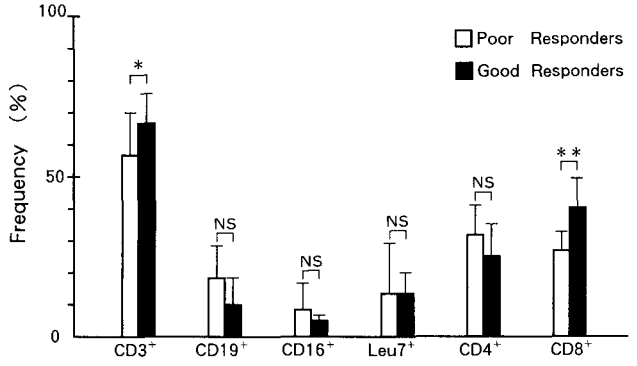

Fig. 3. The phenotypic structure of TIL in each group determined by single-colour analysis. The frequency of $CD3^+$ cells in GR was significantly higher than that in PR (*: $P < 0.05$); however all the frequencies of $CD19^+$, $CD16^+$, and $Leu7^+$ cells in GR were not significantly different from those in PR. Major subpopulations of T-cells such as CD4 and CD8 were examined. Frequency of $CD4^+$ cells in GR did not significantly differ from that in PR, but $CD8^+$ cells in GR were significantly higher than those in PR (**: $P < 0.01$). *NS*, Not significant. *Columns* show means; *bars* indicate $\pm$ SD

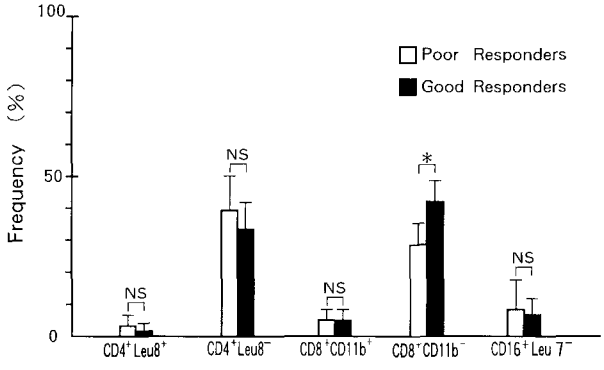

Fig. 4. The phenotypic structure of TIL in each group determined by two-colour analysis. The frequency of $CD8^+CD11b^-$ in GR was significantly higher than that in PR (*: $P < 0.025$). *NS*, Not significant. *Columns* show means; *bars* indicate $\pm$ SD

Detailed subpopulation of $CD4^+$, $CD8^+$ and NK-cells was determined with two-colour analysis. Co-expression of Leu8, CD11b and Leu7 with $CD4^+$, $CD8^+$ and $CD16^+$ cells respectively was examined in 15 cases (GR: 6, PR: 9) in order to delineate subgroups (Fig. 4). The average frequencies of $CD4^+Leu8^+$ cells (Ti), $CD8^+CD11b^+$ (Ts), and $CD16^+Leu7^-$ (most potent NK-cells) of TIL in GR were very low and amounted to $1.1 \pm 1.0\%$, $3.7 \pm 4.9\%$, and 4.7 ± 3.2 respectively, all of which were similar to those of PR ($2.5 \pm 2.6\%$, $3.7 \pm 3.7\%$, $6.8 \pm 9.1\%$) respectively. The frequency of $CD4^+Leu8^-$ cells (Th) of TIL in GR was $28.5 \pm 9.3\%$, which was slightly lower than that in PR ($36.2 \pm 7.1\%$) but was not significantly different. However, the frequency of $CD8^+$ $CD11b^-$ cells (Tc) of TIL in GR was high and amounted to $37.6 \pm 8.6\%$, which was significantly higher than the frequency in PR, which amounted to $25.3 \pm 8.5\%$.

Absolute number of $CD3^+$, $CD8^+$, and $CD8^+CD11^-$ cells, of which frequencies in GR proved to be increasing compared with those in PR, per gram of tumour weight were calculated. The mean numbers of them in GR ($CD3^+$: $(2.21 \pm 0.94) \times 10^6$/g, $CD8^+$: $(1.43 \pm 0.89) \times 10^6$/g, $CD8^+CD11b^-$: $(1.33 \pm 0.82) \times 10^6$/g) showed remarkable and significant increase compared with those of them in PR ($CD3^+$: $(5.00 \pm 4.98) \times 10^5$/g, $CD8^+$: $(2.22 \pm 2.06) \times 10^5$/g, $CD8^+CD11b^-$: $(2.43 \pm 2.33) \times 10^5$/g; $P < 0.005$ for all of them).

The mean $CD4^+/CD8^+$ ratio of TIL in GR was 0.81 ± 0.57, which indicated predominance of $CD8^+$ cells and was significantly lower than that in PR (1.33 ± 0.51; $P < 0.025$), which indicated predominance of $CD4^+$ cells. Mean $CD4^+Leu8^-/CD8^+CD11b^-$ ratio in

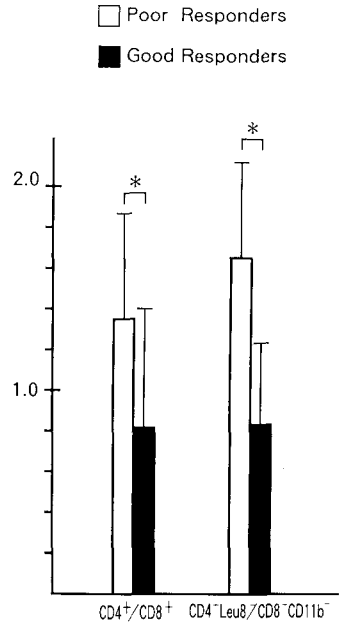

Fig. 5. $CD4^+/CD8^+$ and $CD4^+/Leu8^-/CD8^+CD11b^-$ ratios of TIL. These two ratios in GR are reversed and significantly lower than those in PR respectively (*: $P < 0.025$). *Columns* show means; *bars* indicate $\pm$ SD

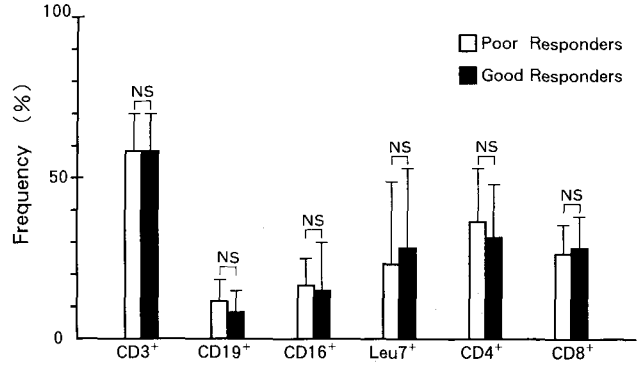

Fig. 6. The phenotypic structure of peripheral blood lymphocytes (PBL) in each group determined by single-colour analysis. No single frequency of major subpopulations of PBL in GR was significantly different from that in PR. *NS*, Not significant. *Columns* show means; *bars* indicate ± SD

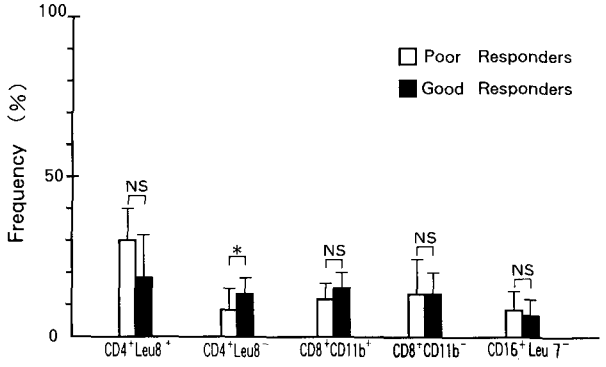

Fig. 7. The phenotypic structure of PBL in each group determined by two-colour analysis. Only the frequency of $CD4^+Leu8^-$ cells in GR was significantly higher than that in PR; however the frequencies of other subsets in GR were not different from those in PR. *NS*, Not significant. *Columns* show means; *bars* indicate ± SD

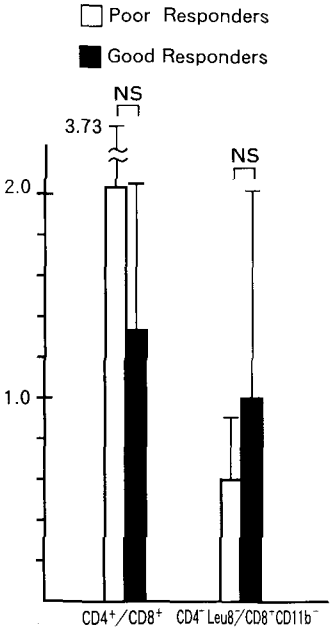

Fig. 8. $CD4^+/CD8^+$ and $CD4^+Leu8^-/CD8^+CD11b^-$ ratios of PBL. These two ratios were not significantly different from those in PR, and $CD4^+/CD8^+$ ratio of both two groups exceeded unity. *NS*, Not significant. *Columns* show means; *bars* indicate ± SD

GR was 0.82 ± 0.40 which was significantly lower than that in PR (1.61 ± 0.46) (Fig. 5).

The mean number of PBL in PR and GR was 1960 ± 731 and 1968 ± 508/mm^3 respectively (no significant difference). Figures 6–8 show mean values or ratios with SD of each phenotype. Not a single frequency of phenotype of PBL in GR determined by single-colour analyses was significantly different from that in PR. By two-colour analyses, only the frequency of $CD4^+Leu8^-$ cells in GR was significantly higher ($P<0.05$) than that in PR. The frequencies of other subsets and ratios of any two subsets in GR were not different from those in PR.

Discussion

We have demonstrated, using quantitative flow-cytometric measurements of the frequency of each subpopulation, phenotypic alterations of TIL and PBL which correlate with the degree of local host response of oesophageal cancer patients. The increasing frequencies of subpopulations of TIL in GR result in an actual increase in number in GR when compared with PR.

Of TIL in GR, the frequencies of $CD3^+$ cells, which were the majority in TIL, were significantly higher than that in PR. However, the frequencies of all the phenotypically NK-cells ($Leu7^+$, $CD16^+$, and $CD16^+Leu7^-$) amounted to a very low percentage and were not higher than that in PR. These results suggest that T-cells may play greater part than NK-cells in the local response to human oesophageal cancer. We also showed that the frequencies of $CD8^+$ cells of TIL in GR, which mainly consisted of $CD8^+CD11b^-$ cells, were significantly higher than those in PR. The results produced $CD4^+/CD8^+$ and $CD4^+Leu8^-/CD8^+CD11b^-$ ratios in GR to be significantly lower than those in PR. Since those ratios were lower than unity, $CD8^+$ and $CD8^+CD11b^-$ were predominant among them. From our results, we can say that $CD8^+$ cells, on most of which CD11b are not expressed, may play a key role in in vivo functions of TIL and in determination of the degree of local response. These findings and our previous clinico-pathological observations, which show that GR have a better prognosis compared with PR (Shiozaki et al. 1983, 1987), together may indicate that the $CD8^+CD11b^-$ cells mainly exert a potentially protective immune host response against tumour cells in TIL. This observation, moreover, is supported by our recent report that $CD8^+CD11b^-$ subpopulation is one of the two subpopulations found to be increased in TIL when compared with controls (Tahara et al. 1990).

Previous reports of the phenotypic characteristics of TIL in animal models and in some other types of human cancer have already shown evidence that T-cells may play a key role in anti-tumour response (Shimokawara et al. 1982). However, it is still unclear and controversial what specific subpopulation is responsible in T-cells. Bilik et al. (1989) recently reported that 66.1% of primary breast cancers have TIL in reversed ratio of $CD4^+/CD8^+$ ($CD4^+/CD8^+<1$) resulting from a decrease in

the number of $CD4^+$ and that there were correlations between $CD4^+/CD8^+$ ratios and numbers of T-cells. Our results in single-colour analysis correspond with their conclusions except for the cause of the reversed ratio. Since this inconsistency might indicate the existence of organ specificity in TIL composition, further observations are required in other organs to certify the hypothesis.

Since $CD8^+$ cells are primarily class I MHC reactive (Meuer et al. 1982), our results in TIL may indicate that the identification of class I antigen by the host at a tumour site may affect the degree of local host response in human oesophageal cancer strongly. These alterations of phenotypic structure in TIL are possibly influenced by the general immunocompetence of patients and we therefore analysed some factors which are related to this. We found no significant difference between GR and PR in mean age, sex, extent of disease, and histological type, all of which might correlate to general immunocompetence of the host (Eilber and Morton 1970; Gupta and Good 1979; Coppola and Tirelli 1985; Paoli et al. 1988). In the number and phenotypic structure of PBL, which reflect general immunocompetence more directly (Reinherz et al. 1978, 1980; Carney et al. 1981), only the frequency of $CD4^+Leu8^-$ cells in PR was significantly lower when compared with those in GR. From these results, we can suggest that general immune status is not a major determinant for local response in operable patients and that the identification of class I MHC antigen by TIL is mainly influenced by local environmental factors. We did not examined the nature of tumour cells in this study, but Smith et al. (1989) reported loss of HLA-A,B,C allele products and lymphocyte function-associated antigen 3 in colorectal neoplasia. Our findings indicate that this phenomenon may also occur in oesophageal cancer.

Great caution, of course, must be used in assessing the relationship between the lymphocyte phenotype and its function. Therefore, $CD8^+CD11b^-$ cells in TIL cannot simply be assumed to have cytotoxic function at a tumour site; indeed, some studies have even pointed out a suppressor function of crude TIL (Vose 1982; Holmes 1985; Miescher et al. 1986; Whiteside et al. 1986). However, the fact that TIL, expanded with IL-2, have enhanced in vitro cytotoxicity against tumour cells when compared with PBL expanded in the same way (Rabinowich et al. 1987; Rosenberg et al. 1986; Takagi et al. 1989) implies a potential function for TIL. Further functional analysis of TIL should be performed with simultaneous analysis of lymphocyte phenotypes considering the remarkable alteration of TIL composition shown by this study. Among many lymphocyte subpopulations, $CD8^+CD11b^-$ should be especially noted in this kind of study in aiming towards development of effective AIT.

In conclusion, $CD8^+$ cells (Tc/s), especially $CD8^+CD11b^-$ cells (Tc), may play a key role in local host response. The identification of class I MHC antigen by the host at the tumour site may strongly affect the degree of local host response in human oesophageal cancer.

Acknowledgments. This study was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, Japan (63-2). We thank Dr. Mark Burley for revising the English style; Drs. Masatoshi Inoue and Hirohito Nagaoka for helpful suggestions; Ms Yaeko Naito, Ms Yoko Tsunetoshi, and Ms Junko Sugihara for excellent technical assistance.

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