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Tumor Infiltrating Lymphocytes in HLA^+ and HLA^- Laryngeal Cancer – Quantitative Approach

GRZEGORZ DWORACKI[1], ALEKSANDRA KRUK-ZAGAJEWSKA[2], ELŻBIETA JEŻEWSKA[1], JAN SIKORA[1] and JAN ŻEROMSKI[1]

[1] Department of Immunology, [2] Department of Otolaryngology, University Medical School, Przybyszewskiego 49, 60-355 Poznań, Poland

Abstract. In search of factors governing the accumulation of tumor infiltrating lymphocytes (TIL), frozen sections from fresh surgical specimens of laryngeal carcinoma (n=36) were tested by alkaline phosphatase–anti-alkaline phosphatase (APAAP) immunohistochemistry for monomorphic determinants of HLA class I and class II expression on tumor cells and for the distribution of lymphoid cells bearing CD differentiation antigens. Cell subsets were quantitated in two tumor compartments, tumor mass and tumor stroma, by computer-assisted image analysis. In a portion of examined samples lymphoid cell suspension was isolated from cancerous tissues and assessed by flow cytometry. It has been found that T cells, localized mostly in tumor stroma, were predominant cell population in the tumor microenvironment. Their ability to penetrate tumor mass but not tumor stroma, by $CD8^+$ T cells in particular, but also by natural killer (NK) cells, was associated with HLA class I antigen expression on tumor cells. In flow cytometric analysis activated T lymphocytes ($CD3^+DR^+$) were abundant in HLA^+ tumors as compared to HLA^- ones. In 4 year follow up of 20 patients the mortality was higher in HLA^- group but the data were not statistically significant. These results show that HLA class I expression on tumor cells favor penetration of cytotoxic lymphoid cells into tumor mass, at least in the laryngeal cancer.

Key words: laryngeal carcinoma; HLA antigens; tumor infiltrating lymphocytes; APAAP reaction, image analysis; flow cytometry.

Introduction

Tumor infiltrating lymphocytes (TIL) are now a well recognized agents of putative local immune response against human cancer. There are several reports in favor of a positive correlation between the intensity of lymphoid aggregates at the site of primary tumor growth and prognosis[25, 31]. There were attempts and clinical trials to grow, propagate and expand TIL *in vitro* in order to reinfuse them to cancer patients as a regimen of specific anti-tumor treatment, albeit with doubtful success[5, 18]. On the other hand, in some human cancers there are regular huge amounts of lymphoid cells surrounding epithelial tumor foci, such as seminoma or lymphoepithelioma of the tonsil, apparently without significant impact on tumor growth[3, 4]. Thus, the factors influencing the density of lymphoid infiltrates at a tumor site and the role of TIL during tumor growth and progression are evidently far from clear. The agents contributing to the accumulation of lymphoid cells at a tumor area include various cytokines produced by tissue macrophages, fibroblasts, granulocytes or tumor cells[21, 27, 29], expression of cell adhesion molecules both, by tumor cells and lymphocytes[1] and finally,

the expression of MHC (HLA class I and II) molecules on tumor cells[6]. The role of MHC molecules in antigen presentation is well known and it is conceivable that the recognition of putative tumor antigens by TIL will be dependent on the proper antigen presentation[11, 16].

Laryngeal carcinoma is an example of human cancer, particularly suitable to study the role of TIL in tumor growth. It is histologically homogenous, almost exclusively squamous cell-carcinoma, often infiltrated by mononuclear, mostly lymphoid cells. Tumor cells lack the expression of HLA class I molecules in a large proportion of cases. Although the prognosis in this cancer is relatively good with the present day modalities of treatment, there are several cases showing a relapse and tumor progression, in otherwise properly treated patients[25, 31].

The aim of the current study was to get better insight into mechanisms governing accumulation and content of TIL in the cancer in question by comparing two quantitative methods – microscopic image analysis of immunohistochemical reactions on tissue sections and flow cytometry on isolated lymphoid cells, both in relation to the expression of HLA on tumor cells. It will be shown that there is a clear correlation between HLA expression and the abundance of some TIL subsets, but only within tumor mass.

Materials and Methods

Patients. The subjects consisted of 36 surgically treated laryngeal carcinoma patients, comprised of 33 males and 3 females, ranging in age from 37 to 74 years. Most of them (n=34) were in the advanced stage of their disease, i.e. T4 or T4, according to TNM classification. In all patients, histological examination of the tumor established the diagnosis of primary squamous cell carcinoma, either keratinizing or non-keratinizing. In 17 patients regional lymph nodes were enlarged, as judged by clinical examination; in 8 patietns metastases were later found in histological evaluation. In the remaining patients enlargement of lymph nodes was classified as reactive changes. All patients were subjected to total or partial laryngectomy. They were not treated by either anti-cancer modality prior surgery.

Tissues. Fresh tissue fragments, both for immunohistochemistry and for flow cytometry, collected directly after surgery, comparised tumor boundaries and its immediate vicinity. For the immunohistology tissue blocks were snap frozen in precooled to –70°C isopentane. Serial 2-cryosections, 6 μm thick, were cut from each tissue block, dried and stored at –70°C. For the flow cytometry, tissue fragments were mechanically minced by means of scissors, without enzyme digestion and subsequently passed through plastic mesh. The suspension of small tissue fragments and released cells in RPMI 1640 tissue culture (TC) medium was overlayered on Ficoll-Uropolin gradient (1.076 g/l) and centrifuged at 3000 rpm for 20 min. The cells collected from the interphase were found to consist of mononuclear, predominantly lymphoid cells of 95% vability, as judged by 1% trypan blue exclusion. The number of cells, sufficient for flow cytometric determination of immunophenotype was obtained from 11 tissue samples only.

Table 1. Monoclonal antibodies used in this study

Designation	CD	Specificity	Source
LCA	45	leukocytes	Dako, Glostrup
Leu-4	3	T lymphocytes	Becton Dickinson
OKT-4	4	helper/inducer	Ortho, Raritan
OKT-8	8	cytotoxic/suppressor	Ortho, Raritan
Leu-11	16	Fcγ RIII, NK cells	Becton Dickinson
Leu-19	56	NK cells	Becton Dickinson
Leu-16	29	B cells	Becton Dickinson
HLA-DR		HLA class II	Dako
sl. 34/28		HLA class I	Dr. M. Trucco, Pittsburgh
A13		TCR γ/δ T cells	Dr. S. Ferrini, Genova

Immunohistochemistry. Alkaline phosphatase–anti-alkaline phosphatase (APAAP) procedure according to CORDELL et al.[9] was used throughout, as previously described[32]. The panel of monoclonal antibodies (mABs), used as primary antibodies, is shown in the Table 1. In brief, serial cryostat sections, after fixation in cold acetone, were incubated with appropriate dilution of respective mAb for 30 min. Following Tris buffered saline pH 7.6 (TBS) wash, sections were subjected to rabbit anti-mouse Ig (30 min), then washed and incubated with APAAP reagent for 45 min (both from Dako, Glostrup). The last 2 steps were repeated, in order to increase sensitivity of the reaction. Finally, sections were treated with AP substrate solution, consisting of Basic New Fuchsin, naphtol AS-Bi phosphate and levamisole, the latter as an inhibitor of endogenous AP (all from Sigma, St. Louis). In the control reactions mAbs were replaced by TBS or by normal mouse serum. The preparations were counterstained with Meyer's hematoxylin and mounted in glycerol jelly.

Flow cytometry. Isolated cells from cancerous tissues were subjected to direct immunofluorescence with a panel of double 2 color (fluorescein and phycoerithrin) fluorochrome labeled mAbs vs. lymphocyte differentiation (CD) antigens comprising IMK Plus (Becton Dickinson) kit (Table 2). Cells (2×10^5 per tube) were incubated with 10 μl of respective mAb in the

Table 2. Percent values of tumor infiltrating lymphocytes (TIL) subsets assessed by flow cytometry and by computer assisted image analysis

Patient's initial	HLA		TIL – cytometry							TIL – *in situ*					
	class I	locus DR	CD3 (%)	CD19 (%)	CD4 (%)	CD8 (%)	CD4/ /CD8	CD3 DR	NK (%)	CD3 (%)	CD20 (%)	CD4 (%)	CD8 (%)	CD4/ /CD8	NK (%)
L.K.	+	–	58.4	36.5	26.7	29.2	0.91	7.3	4.8	86.0	9.0	40.6	51.4	0.79	5.0
J.J.	+	–	74.0	18.0	29.0	45.0	0.64	20.0	6.0	83.4	10.2	23.8	58.8	9.40	63.3
G.J.	+	–	74.7	13.9	46.0	28.7	1.60	43.4	11.7	83.6	12.4	46.7	37.6	1.24	4.0
J.J.	+	–	75.5	18.3	29.5	45.9	0.64	20.4	6.1	83.8	9.9	24.2	53.0	0.46	6.3
Z.T.	+	–	76.5	17.6	47.1	23.5	2.00	8.8	7.1	74.1	17.8	43.2	24.2	1.79	8.0
P.B.	+	+	84.3	11.7	19.6	56.8	0.42	56.8	3.9	0.80	16.0	12.1	50.6	0.24	3.1
N.I.	+/–	–	48.2	48.2	26.5	26.5	1.00	10.2	4.20	90.2	4.8	38.8	50.6	0.77	5.0
B.A.	+/–	–	7.10	25.0	28.0	41.0	0.68	30.00	3.5	53.7	43.8	21.7	29.6	0.73	2.3
B.W.	–	–	55.4	25.6	37.8	37.8	1.00	32.4	13.5	69.5	28.2	35.8	35.4	1.01	2.2
R.J.	–	–	67.86	16.67	39.29	39.29	1.00	47.62	15.48	90.0	8.4	50.4	75.7	0.67	1.8
R.F.	–	–	74.7	15.1	26.0	44.5	0.58	27.7	10.0	57.7	36.8	21.8	37.1	0.59	5.3

dark, at room temperature. After extensive wash in PBS, cell acquisition (10^4 cells) was carried out on FACScan flow cytometer (Becton Dickinson), equipped with an argon ion laser with 15 mW of 488 nm excitation.

Evaluation of immunohistochemical results:

An assessment of expression of HLA antigens on tumor cells. Prevalence of HLA antigens, determined by mAbs directed against HLA monomorphic determinants, was assessed semiquantitatively by the division into 3 groups: positive (above 75% tumor area$^+$), partly positive (between 10–75% tumor area$^+$) and negative (lack or less than 10% of tumor area$^+$). In the evaluation of positive reaction the care was taken to exclude necrotic foci, horn pearles and tissue artefacts.

An assessment of lymphoid cell density. Cell density, as a number of cells per 1 mm^2 visualized by respective mAbs, was determined for all cell subsets in the 2 compartments of the tumor microenvironment – within the tumor mass and tumor stroma. Random selection of microscopic fields for image analysis was carried out at constant values of optical magnification ($\times$ 400), light intensity and microscopic diaphragms. Microscopic images were transferred by means of a TV microscopic color camera (Panasonic) and video card to digitalize the analog signal (Videoblaster FS200 Creative) to the PC computer memory. Measured parameters i.e. surface area and the number of positive cells in predetermined compartments were obtained by means of OPTIMAS v. 4.02 (Bioscan) software. Cells were counted using a module of the software allowing to measure the differences between optical density of cells and the background. For each assessed Amb 10 randomly selected microscopic fields were measured. Results of the counting were subjected to statistical evaluation.

Statistical analysis. The significance of differences in cell density for individual cell subsets in 2 tissue compartments i.e. tumor mass and tumor stroma within tumors differing in HLA expression was assessed by means of U Mann-Whitney test. In order to compare the results of TIL counting by means of image analysis and those by flow cytometry Spearman rang correlation coefficient was calculated.

Results

HLA expression on tumor cells

Examples of HLA class I positivity and TIL infiltration in tumor mass and stroma in tumor samples visualized by APAAP immunohistochemistry are shown in Fig. 1. In 11 cases tumor cells were predominantly HLA class I positive, in 11 partly positive, while in 14 negative. The latter constituted 38% of the total cases tested. In the positive tumors one could notice the loss of HLA expression in heratinizing foci, especially in horn pearls. In one tested tumor the expression of both, HLA class I and class II antigens was evident on tumor cells. In 2 cases both, primary tumor and lymph node metastases were available for immunohistology. There were no differences in HLA expression on tumor cells from the primary and/or the secondary tumor; both specimens were classified as partly positive.

Density of cell infiltrates evaluated by microscopic image analysis

T cells and their subsets. Among lymphoid cells, cells infiltrating both tumor mass and tumor stroma T cells, constituted an overhelming majority. Their density in the tumor stroma did not correlate with HLA

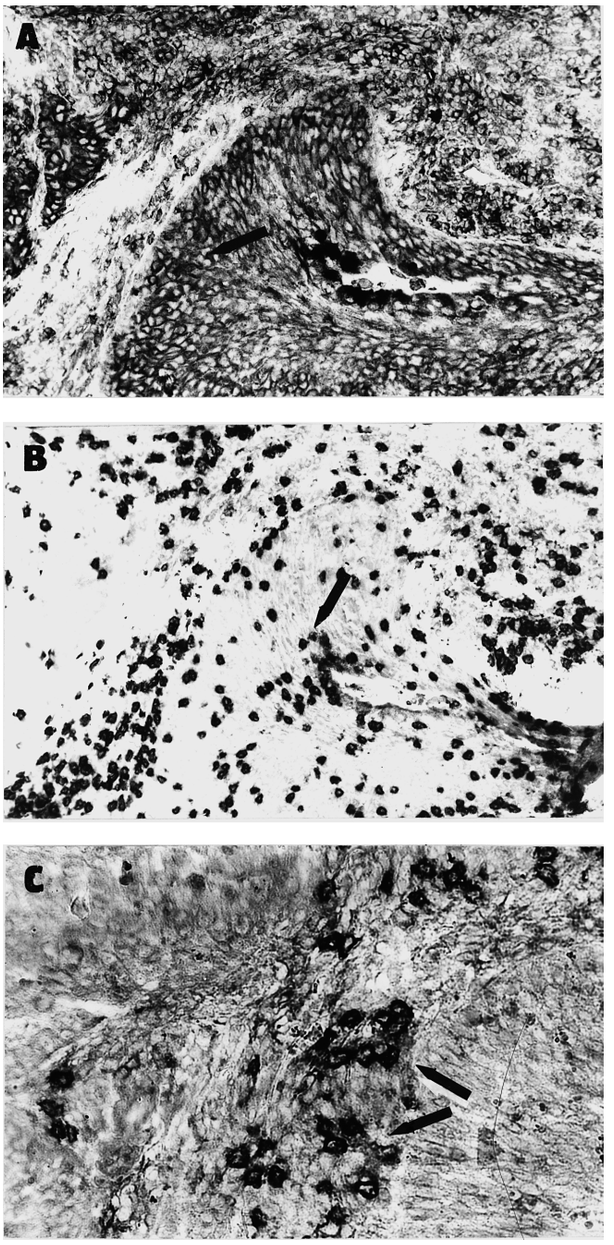

Fig. 1. Subsequent cryostat sections from the same tissue block of HLA^+ laryngeal carcinoma, APAAP reaction. A – monoclonal antibody (mAB) vs. monomorphic determinant of HLA class I antigens. Positive staining of tumor cell membranes (arrows), × 280. B – mAb vs. CD3. Numerous T cells within tumor mass (arrows), × 280. C – anti-CD20 mAb. B cells visible only in tumor stroma (arrows), × 280

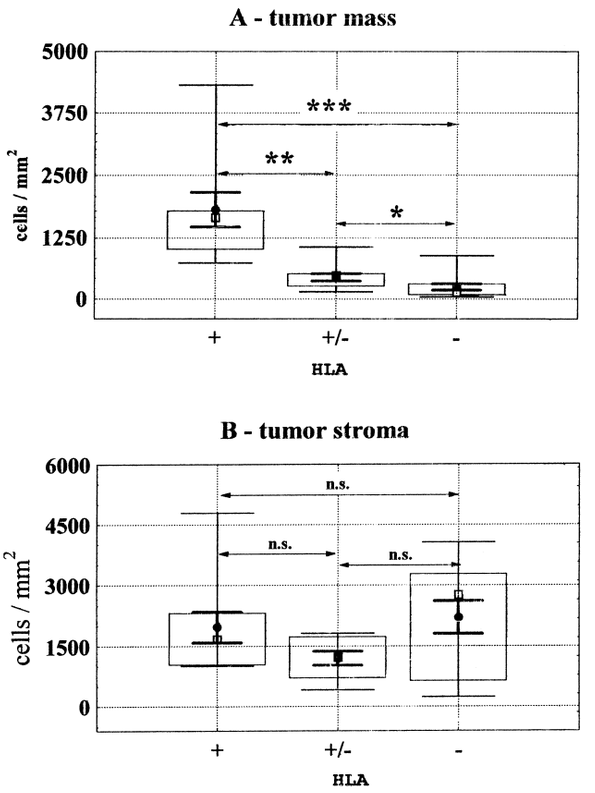

Fig. 2. Laryngeal carcinoma. T cell ($CD3^+$) density within tumor mass and tumor stroma in relation to HLA class I positivity of tumor cells. Computer assisted image analysis of tissue sections. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, n. s. – not significant. Other explanations in the text

expression on tumor cells and was higher than that in the tumor mass, in almost all cases examined. Contrary to this, T cell density in tumor mass was significantly correlated ($p<0.001$) with HLA class I expression on tumor cells. An average density of $CD3^+$ cells in the tumor mass was ± 1818, ± 441 and ± 244 cells per 1 mm^2 for HLA positive, partly postive and negative tumors, respectively (Fig. 2). $CD4^+$ and in particular, $CD8^+$ cells also conformed to this rule (Fig. 3). In the majority of cases $CD8^+$ predominated over $CD4^+$ cells in the tumor mass, while the reverse was true in tumor stroma. This was reflected by $CD^+/CD8^+$ ratio. The latter was lower than 1 in the tumor mass, while higher than 1 in the tumor stroma in most cases (not shown).

T cells with T cell receptor-TCR-1 (γ/δ) were few but also present among TIL in the examined cancer. Their density was low, but higher in tumor stroma than in tumor mass. There was no relationship between HLA expression on tumor cells and γδ cells density.

Other cells. Similar density and pattern of distribution for γδ T lymphocytes was also observed for NK cells. Their density however, has shown noticeable positive correlation with HLA expression, both in tumor stroma and in the tumor mass (Fig. 4).

B lymphocytes ($CD20^+$, $HLA\text{-}DR^+$) were the next most commonly represented among TIL in cancer of the larynx. They tended to form cell clusters and were much

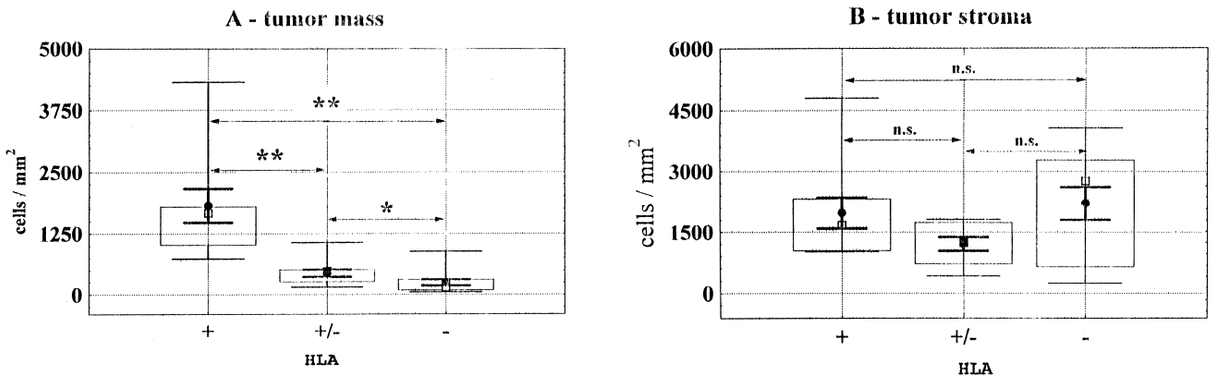

Fig. 3. Laryngeal carcinoma. Cytotoxic/suppressor T cell ($CD8^+$) density within tumor mass and tumor stroma in relation to HLA class I positivity of tumor cells. Computer assisted image analysis of tissue sections. Other explanations see under Fig. 2

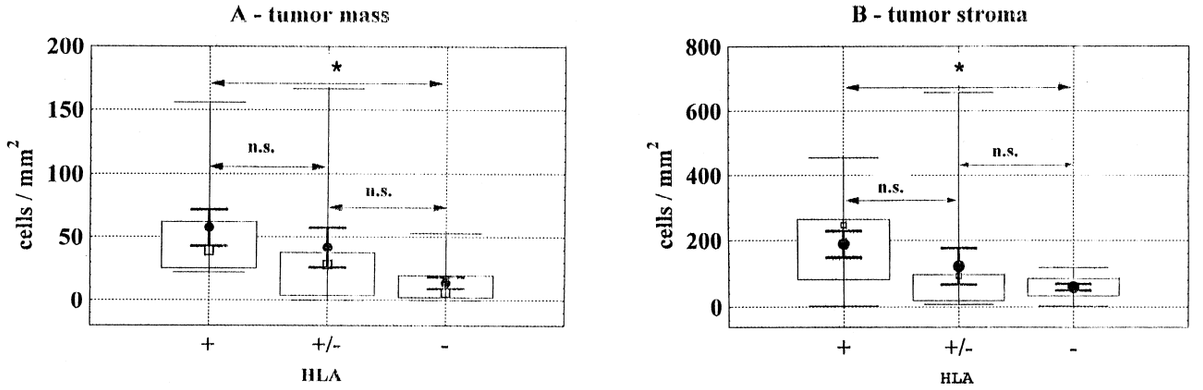

Fig. 4. Laryngeal carcinoma. NK cell ($CD56^+$) density within tumor mass and tumor stroma in relation to HLA class I positivity of tumor cells. Computer assisted analysis of tissue sections. Other explanations see under Fig. 2

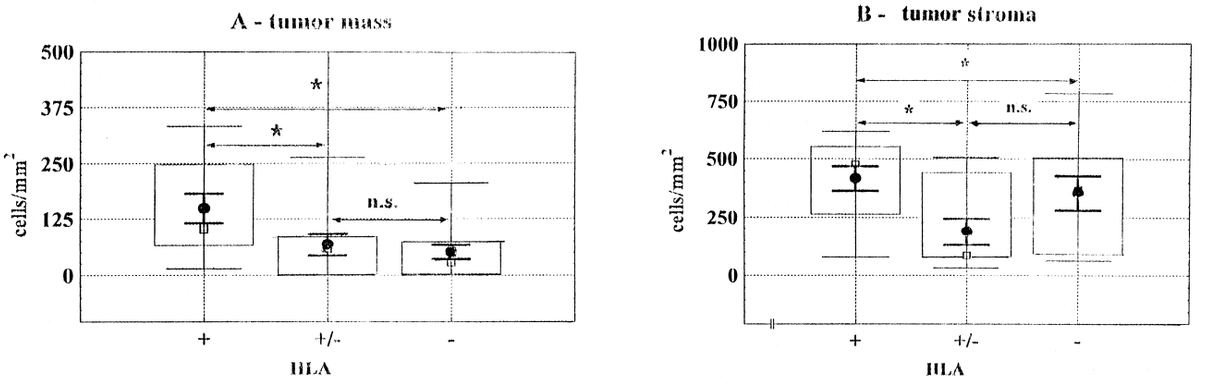

Fig. 5. Laryngeal carcinoma. B cell ($CD20^+$) density within tumor mass and tumor stroma in relation to HLA class I positivity of tumor cells. Computer assisted analysis of tissue sections. Other explanations see under Fig. 2

more frequent in tumor stroma than in the tumor mass, but like NK cells, have shown small but noticeable correlation between cell density within tumor mass and HLA positivity of tumor cells (Fig. 5). It was not possible to discern activated ($HLA\text{-}DR^+$) T lymphocytes from other $HLA\text{-}DR^+$ cells such as B cells, macrophages, dendritic cells etc. among TILs by means of the method used.

TILs evaluated by flow cytometry

Cell samples were subjected to analysis with LYSIS II software, only when the percentage of lymphocytes ($CD45^+$, $CD14^-$) in the gate exceeded 70%. There were 6 cases of HLA class I positive, 2 – partly positive and 3 – negative. In all 11 cases examined T lymphocytes predominanted, ranging from 55 to 84% of the total

lymphocyte pool. The percentage of $CD4^+$ and $CD8^+$ cells varied. $CD4^+/CD8^+$ ratio ranged from 0.42 to 2.0. In the majority of cases it was equal or less than 1. Flow cytometry allowed a clear delineation of activated ($CD3^+$ DR^+). T cells. Their percentage ranged from 7 to 57% of all T cells. The highest (57%) was seen in the case of tumor cells showing the expression of both class I and class II HLA. In the latter case there was also the lowest ratio (0.42) of $CD4^+/CD8^+$ cells. The percentage of NK cells ranged from 3.5 to 15.5 of the total lymphocyte pool, while for B cells these values were 12–48%. It was not possible to assess flow cytometric data in relation to HLA expression due to the low number of cases studied. The lowest percent values of $CD3^+$ cells were, however, found in three HLA^--tumors (Table 2).

Comparison of concordance of two methods used

Spearman's correlation coefficient has shown low values for basic lymphocyte subsets and for CD4/CD8 ratios (Table 3). It was low for 5 examined cell subsets (possible exception were $CD8^+$ cells) and negative for NK cells.

Table 3. Spearman's correlation coefficient R of TIL values from flow cytometry and *in situ* analysis (Table 2)

Parameter	Value
CD3	0.036
CD19/CD20	0.555
CD4	0.176
CD8	0.665
CD4/CD8	0.334
NK	–0.212

Relationships between HLA expression on tumor cells, TILs density and the patients survival

Ten patients died within four years of the postoperative observation period. Of these, their cancer cells were HLA class I negative in 5, partly positive in 3, positive in 2 cases, including one case HLA-DR^+. The information was also available about 10 living patients at that time. HLA status of their tumors as well as CD 45^+ cell density within the tumor mass did not differ significantly from the values of the deceased patients (not shown).

Discussion

The results of this study provide evidence that HLA expression on tumor cells of laryngeal squamous cell carcinoma has a profound effect on the penetration of host lymphoid cells into the tumor mass. The majority of TIL accumulate in the tumor stroma, while cells intermingled with tumor cells are relatively rare in most cases. Obviously host lymphoid cells within the tumor mass, located in direct contact with tumor cells are potential candidates to exert an anti-tumor effect. Several authors suggested such a possibility, following visual evaluation of histological preparations in various cancer types. Abundant lymphoid peritumoral infitrates are generally regarded as a favorable prognostic factor for cancer patients. It is still not quite clear what factors govern migration of TIL toward tumor cells, although the role of cytokines of various origin is generally accepted[20, 30].

Calculated TIL numbers in the current study, obtained with the help of computer-assisted image analysis were generally higher than those reported by other authors. The differences were marked, sometimes tenfold as we have seen numbers exceeding 4000 cells/1 mm^2, which was not the case in the published data of others[16, 17, 22]. A simple calculation suggests, however, that such high cell numbers in tissue sections are feasible. If 1 mm corresponds to 10^3 μm and lymphocyte diameter is roughly 10 μm, it means that a 1 mm^2 may accomodate 10^4 lymphoid cells. This is certainly an oversimplification, but provides indirect evidence for the presumed validity of obtained values.

Expression of HLA class I molecules is diminished or absent in several human solid tumors such as carcinoma of the lung, melanoma, breast and cervical carcinoma and others[8, 10, 14, 15]. This has been also shown in carcinoma of the head and neck, including cancer of the larynx[11, 12]. The diminished HLA expression on tumor cells in usually linked to aggressive tumor behavior. Interestingly, the loss of HLA expression on cancer cells is often associated with markedly decreased or absent local lymphoid cell infiltrates within a tumor area, a frequent finding in solid epithelial malignancies. These accumulations of lymphoid cells, well known as TIL, have been shown occasionally to exert cytolytic activity versus autologous tumor cells *in vitro* in a MHC restricted fashion[19]. On the other hand, some authors were unable to find any association between abundant lymphoid infiltrates and the improvement of prognosis in some cancers. It was later found that such discrepancy may be due to various abilities of lymphoid cells to penetrate into tumor masses from stromal compartments[30].

A clear relationship between HLA expression on tumor cells and the penetration of TIL into a tumor mass as seen in the current study turned out to be possible owing to the precise assessment of the TIL number in both, the tumor mass and stromal compart-

ments, by means of computer assisted image analysis. The relatively homogenous tumor patient group, not treated previously, an access to surgical samples of laryngeal cancer, being exclusively of squamous cell carcinoma type were certainly an advantage for this type of study. Stratification of tumor samples on three subgroups according to HLA class I expression allowed the range of HLA positivity to TIL numbers of the defined phenotype to be compared.

It has been shown explicitly that HLA^+ laryngeal carcinomas show statistically higher numbers of $CD3^+$ T cells within the tumor mass than do HLA^-. It was not the case when tumor stroma in the above mentioned groups of cancer was compared. Mean T cell numbers within the tumor mass in HLA^+ reached 1818 while HLA^- seldomly exceeded 240 per 1 mm^2. Interestingly, $CD8^+$ T cells constituted the majority of cells penetrating the tumor mass, while $CD4^+$ cells were relatively few. It is in line with findings of others who demonstrated that $CD8^+$ cells dominate in bulk cultures of TIL and form major cell population in long term cultures of TIL. To the contrary, NK cells were few, both in the tumor mass and tumor stroma, but interestingly, they have shown some relationship to HLA positivity of the tumor. This may be due to the killer cell inhibitory receptors (KIRs) associated with the allelic pattern of MHC[24]. The latter were not, unfortunately, examined in the current study.

The comparison of TIL searched in tissue sections with those isolated by mechanical dispersion and studied by flow cytometry (FC) has shown advantages and disadvantages of two methods used. In general, HLA^+ tumors were more abundant in T cells while tested by FC. Obviously, it was impossible to discern TIL from the tumor mass and from tumor stroma, using FC. The latter technique allowed, however, to discriminate the activated T cell subset, by visualizing double-stained $HLA\text{-}DR^+$ $CD3^+$ cells. The latter subset was markedly increased in 8 out of 11 cases examined. The demonstration and evaluation of activated T cells in tissue sections is hardly possible, because of the expression of HLA-DR antigens on tissue macrophages and on B ceels. It could be judged only by indirect evidence, as in several cases the cell density of $HLA\text{-}DR^+$ cells was higher than the sum of densities of B cells and macrophages. The latter exceeded 25% of tumor infiltrating cells.

The comparison of per cent values of cell subsets in the group of patients examined, both, by FC and image analysis of immunohistochemical specimens has shown a concordance of some values, especially in CD3 and in CD4/CD8 ratio. In others differences were significant, such as those of B cells. It is conceivable, because conditions of cell dispersion and cell isolation for FC favor accumulation of some cell subsets[23]. B lymphocytes tend to form lymphoid nodules in tissue compartments, which certainly may influence quantitative evaluation.

Special attention should be paid to one case of laryngeal carcinoma whose cells were both HLA class I and class II positive. TIL density in this case was one of the highest out of all cases tested. HLA-DR positivity of tumor cells is regarded prognostically facorable[2]. DR expression may be linked to putative tumor antigen presentation to T lymphocytes, which may explain their massive penetration into the tumor mass. It is known however, that professional APC, apart from HLA-DR expresses B7 molecule binding to CD28 ligand on T cells. The lack of this molecule on tumor cells may severely impede antigen presentation or even induce T cell anergy[7].

The significance of HLA positivity and TIL density for patient survival could not be systematically studied because of relatively short observation period and the low number patients available for observation in the current study. It was however of interest, that in 4 of 5 deceased patients, 2 years after surgery, the tumor cells were HLA negative and TIL densities, especially of $CD3^+$ cells were low, but only in the tumor mass. This coincidence was not however seen after 4 years of observation, when both living and dead patient groups were compared. This is in line with the data of ESTEBAN et al.[13] who found no influence of HLA class I expression on tumor cells and TIL density on cancer patients survival in 6–10 years follow up. It suggests that these 2 parameters, namely HLA expression on tumor cells and TIL density have limited value as an accessory, independent prognostic factor in laryngeal cancer. Functional studies discriminating TIL within the tumor mass and within stromal compartment are needed to clarify this issue.

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