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Cellular characteristics of peripheral blood lymphocytes and tumour-infiltrating lymphocytes in patients with gynaecological tumours

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Abstract Immunotherapy of gynaecological cancer with tumour-infiltrating lymphocytes (TIL) or peripheral blood lymphocytes (PBL) has become a valid treatment modality with varying degrees of success in obtaining an antitumour response. TIL consist of lymphocytes, mainly T cells and minor populations of natural killer cells or B cells. Conventional cytogenetic studies of tumour cells from patients with breast and ovarian cancer have shown multiple chromosomal abnormalities including chromosomes 7 and 12. This study was designed to analyse the surface further, as well as investigate the intracellular, characteristics of TIL by multicolour flow cytometry and the cytogenetic features by fluorescence in situ hybridization. Tumour cell, peripheral blood and TIL samples from 25 patients (15 ovarian tumours, 8 breast cancers, 1 uterine sarcoma, 1 cervical carcinoma) were analysed for their phenotype, the expression of major cytokines [interleukin-2 (IL-2), IL-4 and interferon γ (IFNγ)], their proliferation rate, their cytotoxic ability and for the presence of numerical aberrations of chromosomes 7 and 12. All the tumour cells showed a high frequency of numerical aberration in chromosomes 7 and 12, especially trisomies or tetrasomies and combined aberrations. Trisomies of both chromosomes also occured at a low percentage in TIL and PBL.

The phenotyping of TIL and PBL revealed rather similar subsets of lymphocytes. In both, T cells were the major population, with TIL containing a slightly increased CD4/CD8 ratio. The cytokine pattern showed a predominance of IL-4 production in TIL and of IFNγ in PBL, indicating that, in TIL, cellular immunity is downregulated, whereas in PBL the cytotoxic immune response predominates. This is in accordance with the cytotoxic ability of TIL, which is weakened in comparison to PBL. Cellular characteristics revealed some disadvantages in the use of TIL for cancer treatment, explaining ineffective clinical results. The search for specific antitumour lymphocytes requires carefully designed experiments in order to define effective anticancer cells and thereby improve immunologically mediated tumour therapy.

Key words Gynaecological Cancer · TIL · Cellular characterization · Interphase cytogenetics

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Introduction

Over the past decade immunotherapy has become a valuable tool in the treatment of cancer patients and it is well established that immunological parameters play an important role in host tumour interactions. Therefore, enhancement of the antitumour activity of immunocompetent cells may play a potential part in a great variety of cancer therapies.

Approaches to the stimulation of endogenous host immune cells by active immunotherapy have been supplemented by treatment with lymphokine-activated killer (LAK) cells. This latter form of treatment relies on ex vivo stimulation and propagation of antitumour killer cells. Different sources of these cells have been used. Some protocols describe LAK cell preparation by activation of peripheral blood lymphocytes (PBL) [15, 33, 45, 48] and others prefer tumour-infiltrating lymphocytes (TIL) [8, 16, 32, 36] because of their putative antitumour activity.

TIL consist of lymphocytes, mainly T cells and minor populations of natural killer (NK) cells or B cells. It is tempting to speculate that these cells are effectively conditioned to be antitumour cells, but this is not yet proven [54].

LAK cells have been generated in vitro by incubating TIL with recombinant interleukin-2 (rIL-2) for 6 weeks, in order to enhance tumour-specific cytotoxicity, and then reinfused into the patients in combination with chemotherapy and tumour cell vaccination [37, 50]. Some clinical results with LAK TIL therapy are encouraging [3, 16, 36,

41] but still do not fulfil the expectations raised by theoretical reasoning.

Furthermore, conventional cytogenetic studies of tumour cells from patients with breast and ovarian cancer have shown multiple chromosomal abnormalities involving chromosomes 1, 3, 5, 6, 7, 8, 11, 12, 13, 15, 17, 18 and X [9, 12, 40, 51].

It is not yet known whether TIL are a cell population that participates in the immune response against the tumour, or whether these cells might even enhance the development of the tumour.

Therefore, the aim of our study was to analyse, in more detail, the important characteristics of TIL, in comparison with the corresponding PBL. As a first step, we determined the cytogenetic status using fluorescence in situ hybridization (FISH) and, additionally, we investigated immunological features by flow cytometry, cytotoxicity and proliferation assays.

Materials and methods

Patients

In this study we included 25 adult patients with a median age of 58.9 ± 13.8 years (range, 35–82). Fifteen had the diagnosis of ovarian cancer, 8 breast cancer, 1 uterine sarcoma and 1 cervical carcinoma (stage, FIGO III or IV). Tumour cell and peripheral blood samples were obtained following informed consent at the time of diagnosis or relapse. Cells were analysed by flow cytometry for the expression of different surface markers and cytokine production. In 9 cases we analysed the centromeric regions of chromosomes 7 and 12 by interphase FISH, using α-satellite-chromosome-specific probes. Controls were peripheral blood samples from normal volunteers to determine the background for each probe (n = 5).

Preparation of tumour cells and lymphocytes

PBL were isolated from fresh blood, taken from patients shortly (1–5 h) after surgery, by Ficoll-Hypaque gradient separation, washed twice in phosphate-buffered saline (PBS) and used immediately.

TIL were collected by centrifugation of ascites (800 *g*, 20 min), resuspended in PBS and separated by a discontinuous Percoll gradient (55%–66%) centrifugation for 20 min. Tumour cells were pooled from the interphases and TIL were collected from the pellet. TIL from solid tumours were extracted by mincing and a enzymatic dissection using Dissociation solution (DCS, Hamburg, Germany) for 2–4 h at 30 °C. After passing through a 60-µm-gauze filter, the cells were separated as described, washed in PBS and used immediately.

Proliferation assay

The proliferation rates were determined by the ICA-100 immunocompetence assay (DCS, Hamburg, Germany) and performed according to manufacturers' instructions. In brief, lymphocytes were cultivated for 5–7 days in AIMV medium (Gibco BRL, Eggenstein, Germany) supplemented with autologous tumour cells (1:10) or phytohaemagglutinin (PHA; 2.4 ng/ml) as stimulus, or without supplement as a control culture. ATP was extracted and measured luminometrically by incubation with a luciferin/luciferase reagent on a LB953 luminometer (Berthold, Bad Wildbad, Germany). The stimulation index is defined as the ratio of the responder to the control culture counts.

Cytotoxicity assay

In vitro cytotoxicity of lymphocytes was detected by using the non-isotopic europium release assay [35]. The target K562 cells (10^5/ml) were labelled with a europium diethylenetriaminepentaacetic acid (DTPA) complex (0.5 mM europium-acetate, 1.25 mM DTPA in 50 mM HEPES NaOH, 93 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, pH 7.4) at 4 °C for 1 h. Cells were then washed twice with 2 mM $CaCl_2$ in RPMI-1640 medium and three times with medium alone. Effector cells were added in a ratio of 50:1 to 10:1. Incubation was performed in a final volume of 200 µl for 2 h in a humidified 5% CO_2 atmosphere at 37 °C. Maximum and spontaneous release were determined by addition of 150 µl medium or 0.5% Triton X-100 respectively. After centrifugation (300 *g*) a 50 µl aliquot of the supernatant was transferred to 200 µl enhancement solution. Fluorescence was evaluated in a LKB fluorometer (Wallac, Freiburg, Germany) using a 613-nm filter. Cytotoxicity was calculated as:

specific lysis % = 100×(sample counts - spontaneously released counts)/(maximum released counts - spontaneously released radioactivity counts).

Determination of surface markers and intracellular cytokines by flow cytometry

Surface characteristics of TIL were determined by using the following directly labelled antibodies: anti-CD3, anti-CD4, anti-CD8, anti-CD14, anti-CD19, anti-CD16/CD56, anti-CD28 and anti-CD54 (Becton Dickinson, Heidelberg, Germany). A 10 µl sample of a phycoerythrin (PE)-, fluorescein-isothiocyanate (FITC)- or peridinin-chlorophyll-protein (PerCP)-conjugated antibody was added to 10^6 cells in 100 µl PBS, containing 1% bovine serum. Appropriate isotype controls were used to set the amplification and compensation of the flow cytometer. After an incubation time of 15 min at room temperature in darkness, cells were washed twice and resuspended in 500 µl PBS and then analysed with a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.).

Intracellular cytokines were detected by flow cytometry according to the instructions by Jung et al. [22] slightly modified. Cells were stimulated overnight with 10 nM phorbol 12-myristate 13-acetate and 1 µM ionomycin in the presence of monensin (3 µM) in AIMV medium. After washing with PBS, cells were fixed for 10 min in Hank's balanced salts solution (HBSS) containing 4% paraformaldehyde. Afterwards cells were washed with HBSS and resuspended in 100 µl saponin buffer (HBSS supplemented with 0.1% saponin) (all chemicals from Sigma, Deisenhofen, Germany) and 100 µg/ml goat IgG1 (dianova, Hamburg, Germany). After 10 min the buffer was exchanged and cells were incubated with 1 µg/ml cytokine-specific antibody (PharMingen, Hamburg, Germany) for 30 min at room temperature. After two more washing steps, staining was performed by incubation for 20 min with PE-labelled goat-anti-mouse-IgG antibody (5 µg/ml) (dianova, Hamburg, Germany). In order to determine the phenotype of the T cells, the last step consisted of an incubation with anti-CD3-PerCP and anti-CD4-FITC-antibodies as described above.

Fluorescence in situ hybridization

Cells were fixed in 3:1 methanol:glacial acetic acid and stored until hybridization at –20 °C. Slides were incubated at room temperature in 0.1 M HCl with 0.05% Triton X-100 for 15 min and then washed six times: once for 2 min in 2× saline sodium citrate (SSC), once in PBS, once in PBS with 1% formaldehyde for 5 min, twice for 2 min with PBS, and finally once with 2×SSC. Slides were denatured in 70% formamide in 2×SSC at 70 °C for 2–4 minutes, dehydrated in a 70%, 85%, 100% ethanol series, and air dried.

Directly conjugated centromeric probes (CEP) specific for chromosomes 7 and 12 from Vysis (Stuttgart, Germany) were used for interphase FISH (CEP 7 conjugated to Spectrum green and CEP 12 conjugated to Spectrum orange). A 1-µl sample of each probe was mixed with 7 µl hybridization buffer (50% formamide, 2×SSC, 10%

Table 1 Aberrations of chromosome 7 in patients with gynaecological cancer (mean ± SE). *TIL* tumour-infiltrating lymphocytes

Number of signals	Percentage of cells showing aberrations (%)			
	Tumour cells	Peripheral blood	TIL	Control
1	1.7± 0.8	2.0±0.7	2.0±0.7	1.2±0.7
2	44.5±12.7	94.6±2.4	95.2±1.3	98.7±0.7
3	25.6± 8.2	3.1±1.1	2.7±0.8	0.1±0.2
4	13.9± 7.0	0.3±0.2	0.2±0.2	0.0
5	6.7± 4.5	0.0	0.0	0.0
6	5.6± 4.7	0.0	0.0	0.0
7	0.4± 0.3	0.0	0.0	0.0
8	0.3± 0.3	0.0	0.0	0.0
9	0.2± 0.1	0.0	0.0	0.0
10	1.0± 0.7	0.0	0.0	0.0

Table 2 Aberrations of chromosome 12 in patients with gynaecological cancer (mean ± SE)

Number of signals	Percentage of cells showing aberrations (%)			
	Tumour cells	Peripheral blood	TIL	Control
1	1.4± 0.5	2.0±0.6	1.3±0.4	2.7±1.4
2	37.5±12.5	94.2±1.7	94.9±1.0	97.2±1.4
3	33.0± 9.6	3.7±0.9	3.3±0.8	0.1±0.1
4	16.1± 5.5	0.4±0.3	0.5±0.5	0.0
5	5.4± 2.7	0.0	0.0	0.0
6	2.2± 1.2	0.0	0.0	0.0
7	1.4± 1.1	0.0	0.0	0.0
8	1.4± 0.9	0.0	0.0	0.0
9	0.4± 0.1	0.0	0.0	0.0
10	1.2± 0.8	0.0	0.0	0.0

dextran sulphate) and 2 µl distilled water. Probe DNA was denatured for 5 min at 70 °C and applied to each slide. Hybridization was performed overnight at 37 °C in a humidified chamber.

Unhybridized probe was washed off by a series of three post-hybridization washes in 50% formamide in 2×SSC at 45 °C each for 10 min, followed by one 10-min wash in 2×SSC and one 5-min wash in 2×SSC/0.1% NP-40 at 37 °C. The nuclei were counterstained with diamino-2-phenylindole dihydrochloride (DAPI, 0.2 µM in 90% glycerol/10% PBS, pH 8.0). Hybridization signals were counted by hand in 100–500 cells under a fluorescence microscope (Zeiss, Jena, Germany) equipped with a triple filter set (DAPI/FITC/Texas red).

Statistical analysis

Data are expressed as mean (±SE) or as means (±SD) and analysed by using the two-way analysis of variance and other standard methods. The SPSS statistical package was employed for these analyses as well, to generate descriptive statistics of the data.

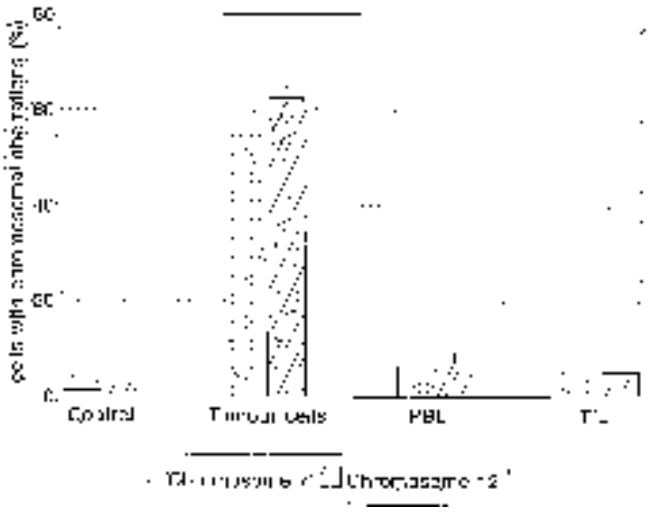

Fig. 1 Aberrations of chromosomes 7 and 12 in tumour cells, peripheral blood lymphocytes (*PBL*) and tumour-infiltrating lymphocytes (*TIL*) patients with gynaecological cancer. The frequency of numerical aberrations in tumour cells was very high. PBL and TIL also carried a very low percentage of trisomies that was still over the background level

Results

We investigated 25 patients with gynaecological cancer. In order to characterize TIL extensively, we combined several methods. In addition, we compared the characteristics of TIL with those of PBL collected from the same patients shortly after surgery.

In 9 patients we analysed the frequency and presence of numerical aberrations of chromosomes 7 and 12 by FISH. TIL from all 25 patients were characterized by fluorescence-activated cell sorting (FACS) analysis using the following surface markers: CD14, CD3, CD4, CD8, CD19, CD16/CD56, CD28 and CD54. In 18 cases PBL were also analysed; 8 patients were investigated by determining cytotoxicity and proliferation index, and in 6 we determined the cytokine pattern.

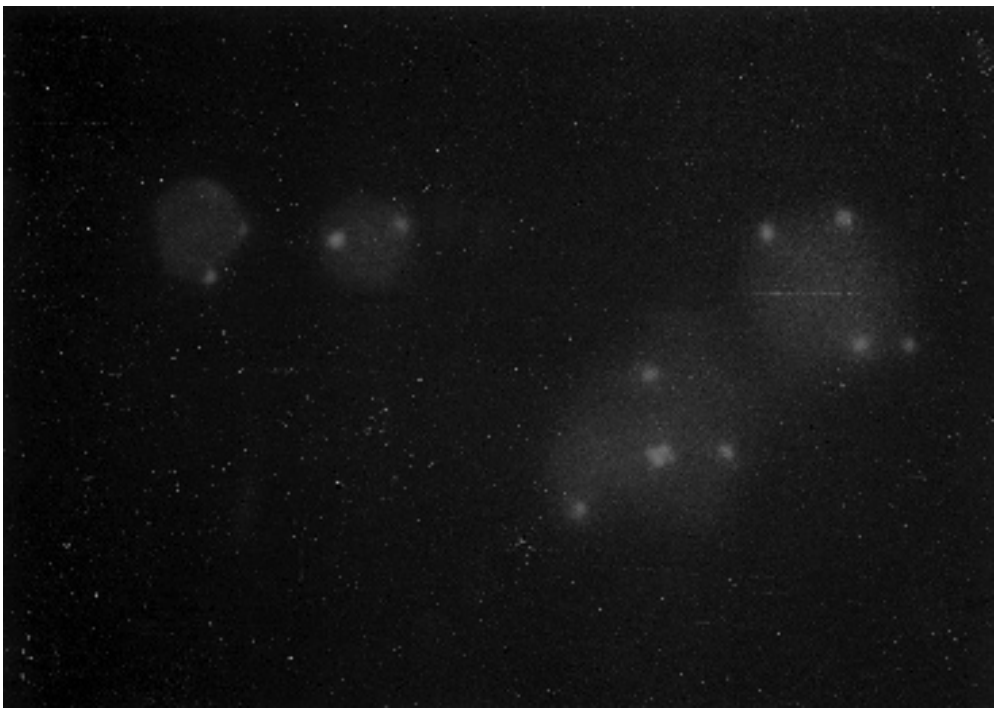

Fig. 2 Two ovarian cancer tumour cells with four signals of chromosome 12 next to two diploid tumour-infiltrating lymphocytes

Table 3 Flow-cytometric analyses of surface markers on peripheral blood lymphocytes (*PBL*) and tumour-infiltrating lymphocytes

Marker	Occurrence in PBL				Occurrence in TIL				Occurrence in control			
	Mean (%)	SE (%)	Range (%)	*n*	Mean (%)	SE (%)	Range (%)	*n*	Mean (%)	SE (%)	Range (%)	*n*
Monocytes												
CD14	14.3	0.8	2–30	16	7.9	0.5	0–55	23	12.8	0.5	2–30	12
Lymphocytes												
CD3	64.4	0.7	33–84	18	71.8	0.6	36–98	27	73.4	0.7	59–83	12
CD4	36.5	0.7	13–56	18	43.5	0.5	22–70	27	46.7	0.7	34–53	12
CD8	28.5	0.7	9–50	18	29.2	0.5	6–53	27	28.0	0.5	18–37	12
CD19	10.4	0.4	2–24	17	8.4	0.4	0–42	25	12.3	0.3	8–17	12
CD16+56	26.3	0.8	6–55	17	15.8	0.5	0–39	25	14.3	0.3	9–25	12
Ratio CD4:CD8	1.5	0.1	0.3–5.2	18	1.9	0.1	0.5–5.3	27	1.8	0.2	1.2–2.6	12
Adhesion molecules												
CD28	44.5	5.0	18–66	4	55.5	4.1	34–69	4	50.5	0.5	32–80	8
CD54	1.0	0.3	0– 3	4	5.0	2.0	0–17	4	17.0	0.3	1–40	8

Fluorescence in situ hybridization

Tables 1 and 2 summarize the percentage of signal gain and loss by chromosome and type of sample. Peripheral blood samples from normal individuals were used as a control for each chromosome probe (n = 5). Tumour cells of patients with breast or ovarian cancers (n = 9) showed mainly trisomies and tetrasomies of chromosomes 7 and 12. We were able to detect 25.6% of cells with three signals and 13.9% of cells with four signals for chromosome 7. Trisomies of chromosome 12 were found in 33.0% of the cells and tetrasomies in 16.1%. On average, 35% of tumour cells carried aberrations of both chromosomes. Figure 1 shows the percentage of chromosomal abnormalities in tumour cells as compared to TIL and PBL. A tumour cell with four signals of chromosome 12 next to a diploid TIL is shown in Fig. 2.

In 3.1% of peripheral blood cells, three chromosome 7 signals were detected. A similar percentage of lymphocytes (3.7%) carried three chromosome 12 signals.

Signal gains of chromosomes 7 and 12 occurred at a very low percentage in TIL similar to PBL (Table 1 and 2). The occurrence of trisomies was very low, but clearly above the background level.

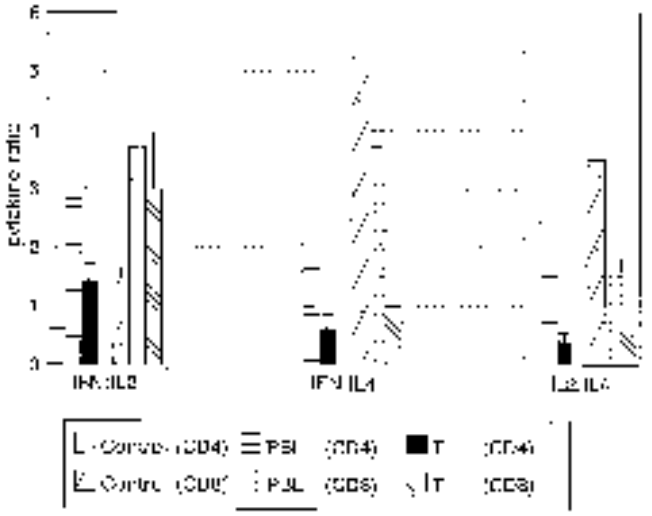

Fig. 3 Expression of intracellular cytokines in lymphocytes. Cytokines were determined by flow cytometry. Data are shown as the ratios of varyious cytokines in different cells

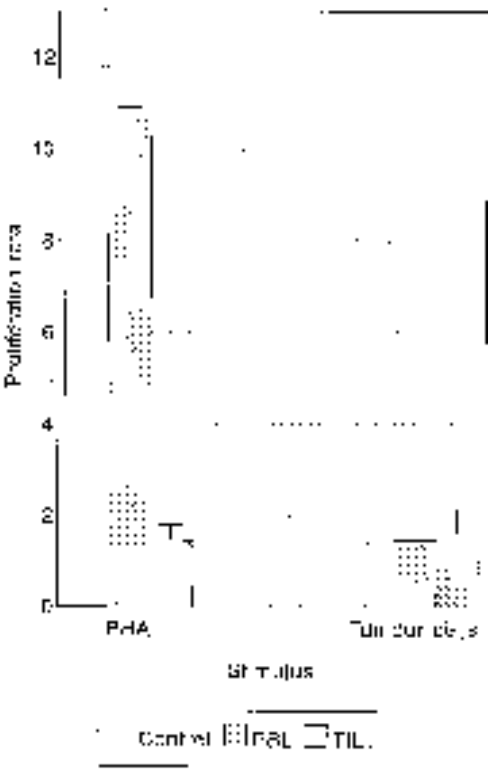

Fig. 4 Proliferation rate PBLand TIL stimulated by phytohaemagglutinin (*PHA*) or autologous tumour cells. PBL showed a 7-fold higher potential in response to the activation by PHA. Tumour cells activated neither PBL nor TIL

Flow Cytometry

FACS analysis of surface markers revealed some differences between TIL and PBL (Table 3). PBL contained normal numbers of monocytes and lymphocyte subsets. They were divided into monocytes ($CD14^+$), T-helper lymphocytes ($CD3^+/CD4^+$), T-suppressor/cytotoxic lymphocytes ($CD3^+/CD8^+$), B lymphocytes ($CD19^+$) and NK cells ($CD16^+/CD56^+$). Very high numbers of monocytes (more than 25%) occurred only in 3 of 16 patients.

TIL subset populations differed from those of PBL in having a higher percentage of T lymphocytes and all other values of mononuclear leucocytes were decreased. We tried to detect monocytes more accurately by using anti-CD-64 antibodies because of the monocyte's important immunological role in antigen presentation, especially in tumour tissue. However, all $CD64^+$ were also $CD14^+$, so that no additional populations of monocytes were recognized. The surface marker characteristics of TIL ranged more widely.

Furthermore, in 13 of 18 patients (72%) the CD4/CD8 ratio of TIL was higher than in the corresponding PBL.

We also studied adhesion markers to detect significant differences between PBL and TIL by measuring the level of expression of CD28 (B7-1 receptor) and CD54 (ICAM-1) as a common adhesion molecule. In TIL, CD54 and CD28 expression as determined by mean fluorescence was slightly increased but there was no significant difference in adhesion molecules between PBL and TIL.

For further information about the physiological function of TIL and PBL we determined the percentage of cells expressing interferon γ (IFNγ), IL-2 or IL-4 (Fig. 3). We calculated the relative ratios of cytokine expression in order to reflect the in vivo situation more accurately, because of the complex biological interaction of cytokines.

Cytotoxic/suppressor T cells ($CD3^+/8^+$) of PBL expressed a high level of IFNγ compared to the other cytokines (mean ratios IFNγ:IL-2 = 3.8 and IFNγ:IL-4 = 3.1). This was the same among PBL T-helper cells, but the results were not so impressive. In PBL T-lymphocytes the IL-2:IL-4 ratio was greater than 1, so that cytotoxic cells predominated. In TIL, IL-4 was the major cytokine expressed, IL-2 the lowest. In $CD8^+$ TIL the IFNγ:IL-4 quotient was almost 1.

Proliferation assays

Proliferation assays were performed to measure the potential of the lymphocytes in response to activation by incubation with PHA as a postive control (Fig. 4). Incubation with autologous tumour cells as activators should be a guide to whether the lymphocytes react to the presence of tumour cells. The activated cells might be capable of finding and reacting against tumour cells and metastases. The proliferation assays revealed a sevenfold higher potential of PBL than TIL in response to the activation by PHA. Incubation with autologous tumour cells activated neither PBL nor TIL so neither lymphocyte population recognized autologous tumour cells.

Cytotoxicity assays

The cytotoxic ability of the lymphocytes was determined by using the europium-release assay against the leukaemia

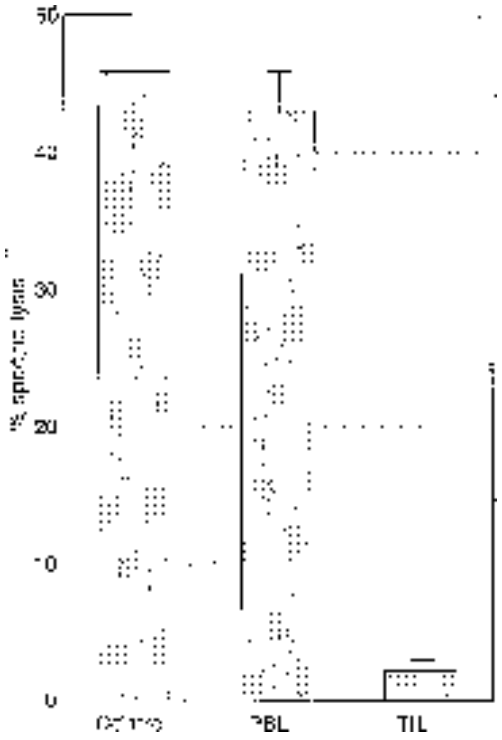

Fig. 5 Cytotoxicitiy assay of PBL and TIL. PBL showed a 19-fold higher cytotoxic ability against K562-tumour cells

K562 cell line (n = 8). The specific lysis of tumour cells by PBL and TIL is shown in Fig. 5. PBL have a 19-folder higher potential to lyse tumour cells than do TIL. In the latter population only a very low number of aggressive cytotoxic cells exist. This result correlates with specific lysis of autologous tumour cells, which were lysed 15-fold more actively by patients PBL than by TIL in all 3 cases investigated (data not shown).

Discussion

The aim of our study was to define the characteristics of TIL from patients with gynaecological cancers and to detect any improvement in immune activity by comparing TIL with PBL from the same patient by several methods.

Conventional cytogenetic studies of tumour cells from patients with breast and ovarian cancer have shown multiple chromosomal abnormalities including chromosomes 7 and 12 [9, 12, 40, 51]. To confirm these findings we used FISH to investigate the frequency and presence of numerical aberrations of both chromosomes in tumour cells and also in TIL and the corresponding PBL, expecting TIL to be aberrant as well.

Trisomy 7 and 12, as well as tetrasomies of both chromosomes and combined aberrations, have been identified by FISH in a substantial number of breast and ovarian tumour cells. Our data now show that TIL are also carriers of trisomies. However, the percentage of TIL with three signals for chromosome 7 or 12 was very low. It is tempting to speculate that these lymphocytes are unable to attack the tumour. Dal Cin et al. [12] have also described chromosomal aberrations such as trisomy 7 and 10 in TIL of patients with kidney tumours. We also observed trisomies of chromosomes 7 and 12 in PBL of patients with breast or ovarian cancers, the frequency of these aberrations being similar to these in TIL.

Phenotyping of both cell types revealed an familiar subdivision of lymphocytes. TIL, like PBL, consisted mainly of T cells expressing the CD3 antigen. These findings are in agreement with other studies of cancer tissues, for example breast [5, 10, 11], ovary [13], lung [57] and melanoma [29]. However, further subset analysis of the lymphocytes led to the identification of some differences. Many investigators have described a variable but low concentration of NK cells ($<25\%$). In TIL from patients with colon cancer, $CD56^+$ cells were even higher than in corresponding PBL [10, 11, 25, 55, 57]. Our results confirm these previous observations. In only 2 of 17 cases studied were the NK cell levels higher in TIL than PBL and only 4/25 TIL populations contained more than 25% $CD16^+/CD56^+$ lymphocytes. This may indicate a subordinate role for NK cells or a need for more NK cells in cancer defence.

As a possible mechanism for immunotherapy, the T cell population, composed of a mixture of T-helper and T-cytotoxic/suppressor cells (indicated by the CD4/CD8 ratio) is more intriguing [54]. In our study the ratio of T cells revealed a higher proportion of T-helper cells. The ratio was predominantly 1 or more in PBL (66%) and TIL (72%). Furthermore, in 13/18 cases TIL showed a higher CD4/CD8 ratio than the corresponding PBL. These data contradict the findings that the majority of TIL T cells are $CD8^+$, resulting in a lower CD4/CD8 ratio in comparison to PBL. This especially is the situation in gynaecological tumours such as breast cancer [5, 11] and ovarian carcinoma [21] and even in melanoma [29] and lung cancer [42, 57] but tumours of the colon are an exception [55]. However, up to now it has not been clear whether therapeutically useful T cells should be mostly $CD4^+$ or $CD8^+$ cells. T-helper cells play an important role in tumour regression mediated by the immune system [18, 29], but tumour-lysing cells are often identified as being CD8-postive [5, 16].

Additional information was collected by investigating the adhesion molecule expression of lymphocytes. We concentrated on two potent T-cell-stimulation systems, CD54 (ICAM-1) and CD28 (B7-1-receptor). ICAM-1 is reported to play an important role in recognition and subsequent stimulation of CD4 cells [18]. Its expression in TIL (up to 30%) from lung cancer tissue is reported to be significantly higher than in PBL (below 5%) of these patients [42]. We also observed that the CD54 expression in TIL is higher than in PBL, but the total amounts of 5% and 1% respectively are indicative of a decreased function of the ICAM system in our patient population. If ICAM-1 is expressed on tumour cells, it should result in greater lysability [1, 6], but this remains to be detected in gynaecological tumour cells.

We also detected CD28 molecules on our lymphocyte samples. This responder molecule is thought to be constitutively expressed [28] in order to receive activation signals and may be able to substitute CD3 in the activation and expansion processes of TIL [58]. Our data (PBL: 44% and TIL: 55%) are in agreement with the data from the control group (51%) and do not suggest that lymphocytes uniformly develop the features of additional activation.

Proliferation is also one of the important properties of therapeutically useful lymphocytes, because the clinical success is dependent on a large number of reactive cells. Therefore we investigated the proliferation rate of TIL and PBL. Previous reports have shown that the proliferation rate of TIL is much lower than that of PBL in gynaecological tumours [13] and colon cancer [4], though gastric cancer is an exception [25]. Our data suggest that TIL from gynaecological tumours are difficult to expand because of their poor proliferation indexes. Even PHA, a powerful stimulator of lymphocytes, failed to increase their multiplication rate.

Furthermore the putatively useful lymphocytes are described by their cytotoxicity capacity against tumour cells. We observed a 19-fold higher cytotoxicity of PBL in comparison to TIL. This effect did not change after IL-2-stimulation of cultured cells. This might be one reason for the latter's disappointing effect in clinical trials. The low tumour-cell-lysing capacity of gynaecological TIL is in accordance with other observations made on lymphocytes from several sources [56], especially breast [11, 46] and gastric cancers [23, 25].

Additional characteristics of lymphocytes may be defined from their pattern of cytokine expression, which describes the functions of lymphocytes more precisely than phenotyping of surface molecules [27]. IL-4-expressing cells promote the humoral immune response. In contrast IL-2 and IFNγ overlap IL-4 and promote cytotoxic response [39]. The characteristic lymphokine expressed by TIL is thought to be IFNγ [4, 17, 44, 54]. However, our investigations and other reports provide contradictory evidence. We detected IL-4 as the main cytokine and IL-4 and IL-10 are expressed in TIL from renal cell carcinoma [53] and melanoma [24]. In TIL from breast cancer tissue little or no cytokine production is reported [11, 52].

We think the situation is more complicated. The cytokine pattern is helpful in order to subdivide T-helper cells; Th1 cells, which augment the cytotoxic component of the immune system, are characterized by IL-2 and IFNγ production with their counterpart, the humoral limb, promoting Th2 cells, which express IL-4 and IL-10 [18, 38]. Undifferentiated Th0-cells express all cytokines and the balance between IFNγ and IL-4 determines the further development of the subsets [38].

For tumour therapy, it is important to know the TH1/Th2 ratio [18] because of the procedure to propagate specific cells. This might protect the resistance of tumour cells to non-specifically activated lymphocytes, as reported for ovarian carcinoma [38]. In our work, IFNγ production is predominant in PBL, indicating that the cytotoxic part is acting more strongly. The cytokine enhances the expression of MHC, adhesion molecules and processing of antigens [18]. The accumulation of Th2 cells in tumour tissue, indicated by IL-4 production, occurs in renal cell carcinoma [18] and melanoma [24] and is reported here in gynaecological tumours. Inhibition of spontaneous cytokine production and activation of specific cytokine expression as effected by tumour cells is reported for breast and ovarian cancers [26, 52].

Taken together, the minor clinical effect of TIL may be due to their cellular characteristics. PBL from the same patients show the same or even better qualities than the corresponding TIL. The incomplete activation of TIL in vivo may be due to the weak stimulus provided by tumours, the lack of antigen-presenting cells or the tumour-induced accumulation of Th2 cells instead of Th1 cells [18]. Recently, similar findings have been reported for human gliomas [43]. The cause of a partial tumour response might also be due to structural changes in T cell receptors, deficient signal transduction or tumour-released suppressing factors [54]. But the true explanation remains unknown.

Cytotoxic lymphocytes from PBL are known to be an alternative therapeutic tool to TIL [20]. Protocols for their generation have been described for renal cancer [7], melanoma [47], lung cancer [30] and gastric cancer [31]. Precursors for antitumour cells might exist in PBL [47, 49] but they are probably present in low numbers. Therefore they have to be carefully defined and selected by various techniques [14], and identification and culture techniques have to be optimized [2].

Successful lymphocyte immunotherapy depends on factors such as adhesion markers and cytokine expression [19]. Antitumour cytotoxic cells represent a range of functionally distinct subsets [34]. It is still not known whether there is a single method of creating powerful, specific, antitumour-reactive cells, but we conclude from the work of others and our investigations that some potent precursor cells exist in every patient. We need to strengthen the search for these therapeutic tools.

The future characterization of lymphocytes from different sources (PBL or TIL) for clinical trials must be performed by a combination of several different methods. This will make it possible to detect the special tumour-specific and tumour-reactive cells. New protocols of cancer treatment by immunological methods will require this information in order to be successful.

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