Functional analysis of tumor-associated lymphocytes from gynecological tumors

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Summary: Tumor-Associated Lymphocytes (TAL) were isolated from peritoneal fluids of six ovarian cancer patients and pleural effusion from eight breast cancer patients, respectively. In one case we obtained ascitic fluid as well as pleural effusion because of intraabdominal metastatic breast carcinoma. The collected cells were cultured in a complete medium and supplemented with human interleukin-2 (nIL-2) in a concentration of 1000 Units/ml. Phenotyping was not always possible due to rapid decay of the cells. Cytotoxicity was determined with a fluorescence-based assay, in some cases at different stages of cell growth. In two cases TAL from ascitic fluids showed increased cytotoxic activity after a longer cultivation period. TAL from pleural effusions showed cytotoxic activity against the target cell lines in two cases only. Some of these TAL did not proliferate any more but died within 24 h. With the functional analysis we wanted to investigate the cytotoxic potential against natural killer (NK)-sensitive and NK-resistant (Raji) cell lines. The results demonstrate the ability of some of the TAL populations to destroy tumor cells.

Key words: Tumor-associated lymphocytes; Cytotoxic activity; Interleukin-2.

INTRODUCTION

Lymphocytic infiltration in the area of and within a malignant tumor of the breast as well as in ovarian cancer is regarded as evidence of an immunological response to the tumor ([1, 2, 3]). The accumulation of lymphocytes at the tumor site has been considered as a manifestation of immunological recognition of tumor cells by the immune system of the host ([4]). However the contribution of these lymphocytes to host responses to the tumor remains unclear.

In some studies the lymphocytic infiltration of the tumor and the surrounding areas has been associated with improved prognosis and survival ([5, 6]) whereas in some cases human tumor-infiltrating lymphocytes (TIL) failed to proliferate, even in response to high doses of nIL-2 ([7]). It seems probable that the depressed responsiveness of the lymphocytes to cytokines may be due to suppressive influences of the tumor environment ([8]). Also a functional defect of NK-cells resulting in a reduced or non-measurable cytotoxicity as an underlying reason is discussed ([9]).

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Table 1. — *Source of tumor-associated lymphocytes.*

Case	Age	Tumor histology/stage	Time[1]	Metastasis	Time[2]
1	66	le: pT4N2M0G2, solid ri: pT3N2M1G3, solid	38 15	osseous, hepatic, peritoneal, pleural, local, retroperiton.	6 mo
2	64	pT1aN0M0, solid-adenoid	120	pleural, pulmonal, local	> 9 mo
3	71	pT2N1M0, solid	126	pleural, pulmonal, hepatic	> 10 mo
4	47	pT3N2M0G3, solid	8	pleural, hepatic, local	6 d
5	51	pT4N3M0, solid	120	pleural, hepatic, peritoneal	9 d 59 d
6	51	ri: pT2N1M0, solid le: ?	132 72	local, pleural. osseous	6 d
7	47	pT1N×M0G2, solid	19	hepatic, local, osseous, pleural, ocular	4 d
8	37	pT2N1M0G2, solid	31	pleural, peritoneal, local	1 d
9	52	pT3cN1M1G3 FIGO IV, adeno	10	bladder, intestine, local, spleen, liver	> 11 mo
10	50	pT3cN×M0G3 FIGO III, adeno	4	peritoneal, pleural, local	57 d
11	61	pT3cN1M1 FIGO IV, adeno	0	peritoneal, pleural, local	> 9 mo
12	43	pT3cN0M0 FIGO III, adeno	0	peritoneal	> 8 mo
13	67	pT3cN×M1 FIGO IV, adeno/mix	7	peritoneal, bladder, intestine, local, liver	> 11 mo
14	71	pT3cN×M1 FIGO IV, adeno	26	peritoneal, pleural, local, liver	> 4 mo

Cases 1-7 are patients with breast cancer and patients 8-14 are patients with ovarian carcinoma. From patient 13 we had ascites and drainage fluid separately tested for cytotoxic activity.
1 = Time from diagnosis to puncture (months).
2 = Survival after puncture.

In order to evaluate the potential ability of tumor-associated lymphocytes to kill tumor cells in vivo, we performed cytotoxicity assays with standardized NK-sensitive K 562 and NK-resistant Raji cell lines as target utilizing effector cells derived from pleural or ascitic fluids and cultured with nIL-2.

MATERIALS TND METHODS

Patient population

The 14 patients in this study were diagnosed as having either ovarian epithelial carcinoma or metastatic breast cancer, respectively. Tumor diagnosis was proved histologically. Clinical data are listed in Table 1. The patients with ovarian carcinoma ranged in age from 43 to 71 years and the patients with breast cancer ranged in age from 37 to 71 years, respectively.

Analysis of surface markers of TAL

Surface markers of TAL were detected using a direct immunofluorescence assay. The monoclonal antibodies (MAB's) used for flow cytometry were mouse anti-human MAB's either fluorescein isothiocyanate- or phycoerithrine-conjugated (Becton-Dickinson, Heidelberg). They were directed against the following lymphocyte antigens: CD4, CD8, CD3, CD16, CD56, CD2 and CD25. Aliquots of TAL (1×10^6 cells/ml) were centrifuged and resuspended in 50 µl PBS (+0.1% NaN_3). The cells were incubated for 30 min. at 4° C in the dark with 20 µl of each mouse anti-human mab's listed above. Afterwards 2 ml PBS (+0,1% NaN_3) were added and the whole mixture was spun down gain. The supernatants were discarded and 25 µl of a propidiumiodide solution was added to each pellet (propidiumiodide stock solution 50 µg/ml) and incubated for 2 min. The unbound propidiumiodide was washed out with two additional washing steps each with 2 ml PBS.

After the last centrifugation step the supernatant was discarded again and the pellet was resuspended in 350 µl PBS (+0,1% NaN_3). The cells were then stored at 4° C in the dark until flow cytometry was performed.

Preparation of tumor-associated lymphocytes

Tumor-associated lymphocytes were isolated from ascitic fluids or pleural effusions after puncture. The mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation technique. Cells collected at the interface were washed twice in Hank's balanced salt solution (HBSS) and resuspended at a concentration of $2\text{-}4\times10^6$/ml in Dulbecco's modified eagle medium (DMEM) containing penicillin (100 Units/ml), streptomycin (100 µg/ml) plus 10% human heat-inactivated AB serum and human IL-2 (1000 Units/ml). Human IL-2 (nIL-2) was generously provided by Biotest (Dreieich, Germany). TAL from pleural effusions in general could not be cultivated longer than 14 d and so cytotoxicity assays were carried out during that time. TAL from ascitic fluids were tested for cytotoxic activity after 5-18 d of growth, respectively. These incubation times were chosen in comparison to the generation of LAK-cells ([10, 11]) and to measurements of proliferative responses to mitogens already described ([8]).

Tumor lines

The target cells used in this study consisted of K562 erythroleukemia which allow the detection of NK-like activity. A cultured Raji lymphoma line was used for NK-resistant targets.

Cytotoxicity assay

Cytotoxicity was determined by a fluorescence-based assay already described ([12]). Target cells were labelled with europium chelate (eudiethylenetriaminophenacetate) and after cytolysis caused by the effector cells the Eu^{3+} complex was released into the culture supernatant. Afterwards β-naphthoyltrifluoroacetate was added inducing the formation of highly fluorescent chelates which can be measured with a fluorometer. Target cells (5×10^6/ml K562 and 1×10^7/ml Raji) were labelled with Eu^{3+} in labelling buffer containing 50 mM Hepes, 93 mM NaCl, 5 mM KCl, 2mM $MgCl_2+6H_2O$, pH 7.4, and either 50 µM $EuCl_3$ plus 250 µM diethylene triaminopentaacetate (DTPA) for labelling K562 targets or 100 µM $EuCl_3$ plus 500 µM DTPA for labelling Raji targets.

Immediately before mixing the washed target cells and the labelling buffer, 50 µl/ml of a solution containing 10 mg/ml dextran sulphate in distilled water, were added. The cells were incubated in the presence of Eu^{3+} for 30 min. in an icebath, carefully mixing them every 10 min. The reaction was stopped by adding 30 µl/ml of a 100 mM $CaCl_2$ solution. After 5 min. the cells were washed 4 times with DMEM containing 2 mM $CaCl_2$.

During the washes the effector cells were counted, diluted and plated into microtiter plates (100 µl per well). The target cells were diluted to 5×10^4 cells/ml in DMEM containing 10% FCS (Gibco) and antibiotics as indicated above. 100 µl of this suspension were pipetted into the microtiter plates already containing the effector cells. The plates were incubated for 2.5 h at 37° C and afterwards centrifuged. 20 µl of the supernatants were transferred to microtiter strips and 200 µl of enhancement solution (Delfia, Pharmacia-LKB, Finland) were added. The fluorescence was measured in a fluorometer (Delfia, Pharmacia-LKB, Finland). Calculation of the cytotoxicity values was done with the following formula:

$$\% \text{ lysis} = \frac{\text{test release - spontaneous release}}{\text{maximum release - spontaneous release}} \times 100$$

The maximum release (= total release) was obtained from target cells treated with 0.5% Triton X-100. For spontaneous release target cells were incubated without effector cells during the test and the normal release of Eu chelate was measured.

RESULTS

Functional analysis of tumor-associated lymphocytes

Tables 2 and 3 show the cytotoxic activity of TAL from the different fluids after

Table 2. — *Cytotoxic activity of tumor-associated lymphocytes from pleural effusions cultured with nIL-2 (2-14 d).*

Case	E:T	K562	Raji	E:T = Effector: Target Ratio
1	40:1	14.2	1.1	
	20:1	4.8	–0.2	
	10:1	2.5	–2.0	
	5:1	0.5	1.1	
2	40:1	80.7	28.1	Cytotoxic activity of TAL from patient 8 was not determined
	20:1	64.4	14.2	
	10:1	35.9	7.6	
	5:1	21.9	2.4	
3	40:1	69.6	18.2	
	20:1	40.6	8.2	
	10:1	16.6	1.2	
	5:1	7.7	1.2	
4	40:1	–0.4	–1.4	
	20:1	0.04	1.1	
	10:1	–0.08	–0.1	
	5:1	–0.2	0.7	
5 b	40:1	18.1	0.6	
	20:1	7.1	0.1	
	10:1	–	0.7	
	5:1	4.6	0.02	
6 a	40:1	31.3	32.5	
	20:1	19.3	23.2	
	10:1	13.5	11.9	
	5:1	0.3	10.2	
b	40:1	9.0	4.1	
	20:1	6.4	1.9	
	10:1	0.4	–	
	5:1	–3.2	–	
7	40:1	10.0	2.8	
	20:1	4.1	–0.5	
	10:1	4.0	0.8	
	5:1	1.9	–0.9	

incubation with nIL-2 for 2-14 days (pleural TAL) or 5-18 days (ascitic TAL), respectively. In two cases the cells from ascitic TAL could even be activated for 21-35 days and they still showed cytotoxic activity (data not shown). The cases in the tables are identical with the patients and their clinical data listed in Table 1. In Table 2 we only tested TAL from seven patients. TAL of patient 8 could not be tested due to rapid cell death at the second day of cultivation as was verified by dye-exclusion tests with trypan blue.

TAL isolated from pleural effusions showed good cytotoxic activity only in cases 2 and 3. In case 6a the cells from the

Table 3. — *Cytotoxic activity of tumor-associated lymphocytes from ascitic fluids cultured with nIL-2 (5-18 d).*

Case	E:T	K562	Raji		K562	Raji
5a	40:1	28.3	13.5			
	20:1	19.3	2.9			
	10:1	3.8	–6.8			
	5:1	–4.1	–			
9	40:1	100	–			
	20:1	98.8	–			
	10:1	92.2	–			
	5:1	88.5	–			
10	40:1	100	–			
	20:1	100	–			
	10:1	50.4	–			
	5:1	46.1	–			
11a	40:1	0.5	0			
	20:1	0	0.9			
	10:1	–3.3	1.2			
	5:1	–1.8	0.8			
b	40:1	54.4	5.4		60.7	5.3
	20:1	31.5	2.1		34.1	2.8
12d	10:1	16.3	0	18d	16.2	0.06
	5:1	5.0	–0.5		5.5	0.6
12	40:1	40.7	9.2		82.1	8.0
	20:1	18.7	3.8		49.9	1.0
8d	10:1	7.3	3.5	14d	24.3	–1.7
	5:1	1.9	2.6		10.8	–2.7
13	40:1	0.7	1.7			
	20:1	1.2	0.2			
	10:1	0.06	–0.7			
	5:1	0.5	0.0			
14	40:1	0.7	2.3			
	20:1	0.0	0.6			
	10:1	–0.8	1.6			
	5:1	–1.4	1.1			

first pleural effusion showed little function whereas the cells from the second pleural effusion 6b laidly lost any cytotoxic activity. TAL from ascitic fluids showed 100% cytotoxicity in two cases (9 and 10). Even after 35 days in culture they still showed a lysis of more than 90% (data not shown). Case 5a only shows little function whereas cases 13 and 14 did not show any cytotoxic activity at all. In cases 11b and 12 it could be shown that longer activation periods led to enhanced cytotoxicity of the lymphocytes. Phenotyping showed mixed populations primarily consi-

sting of cytotoxic T lymphocytes. We found high amounts of CD3, CD8 and CD4 populations whereas CD16 and CD56 were only expressed in small numbers. Some of the TAL almost lost the expression of surface markers showing a good correlation with the loss of cytotoxic activity. Due to the low cell numbers and/or the rapid decay of some of the lymphocytes analysis of the subsets was not always possible.

DISCUSSION

The prominent cellular component of mononuclear cells (MNC) found in and around many human solid tumors is represented by tumor-infiltrating and tumor-associated lymphocites ([13, 14]).

Their role in influence on growth and metastasic spread of cancer has not been established. Both cell types cultured in 1000 U/ml of nIL-2 yield heterogenous mixtures of effector cells that do not differ substantially from lymphokine-activated killer (LAK) cells concerning their phenotypic and functional characteristics ([3, 15, 16]). Regarding the functional activities of TIL it has been reported that they show reduced immunological responses and poor cloning frequencies probably due to the effect of putative tumor-derived factors ([8, 15]). Furthermore it has been shown that TIL obtained from metastatic tumors are generally less functionally active than those from primary tumors ([15, 17]).

It has been suggested that tumor-derived inhibitory factors, such as transforming growth factor β (TGF-β), prostaglandin E_2 (PGE_2) or prostacyclin (PGI_2) and thromboxane could be responsible for this inhibition ([18, 19]). For patients with breast cancer, transforming growth factor transcripts determined in biopsies were even suggested as a marker for prognosis ([20]).

Functional analysis of tumor-associated lymphocytes and their response to IL-2 may predict good or bad prognosis. Previous reports already suggested the prognostic significance of autologous tumor-killing tests in cancer patients ([21, 22]).

The potential of PBL to kill autologous tumor cells predicted a favourable clinical course in patients with localized lung cancer ([21]).

In patients with breast cancer and in patients with epithelial ovarian carcinoma, PBL were tested for their ability to selectively lyse autologous tumor cells in comparison with tumor-infiltrating lymphocytes and their functional properties ([23, 24]). Our results indicate different functional states for the TAL from patients with breast cancer and ovarian carcinoma, respectively, even though we did not use autologous tumor cells.

TAL from pleural effusion only showed cytotoxic activity in two cases whereas the loss of function of the others correlated with a poor prognosis and a bad clinical course.

For TAL from ascites we found the same correlation between the loss of expression of surface markers, cytotoxic activity and clinical course.

Table 3 shows the highest cytotoxic activity for cases 9 and 10. Cases 11a and b are ascitic fluids taken at different times. 11a was taken from the patient during operation and 11b one week later. After 18 days of cultivation case 11b showed enhanced values concerning functional activities. In case 12 a longer culture period also led to enhanced cytotoxicity of TAL. These results suggest that the immunosuppressive effects of the tumor cells can be reduced. This reduction could either be due to high concentrations of nIL-2 or to a dilution of the immunosuppressive factors. Thus it appears a probable therapeutic way of stimulating cytotoxic cells of the peritoneal cavity with

nIL-2 by the intraperitoneal route in patients with ovarian cancer. Current research investigating the effects of depressed immune reactivity should be considered in order to achieve the most optimal anticancer therapy.

CONCLUSION

The potential ability of nIL-2 to increase cytotoxic activity of TAL – especially in patients with ovarian cancer – gives rise to the question of this cytokine as a therapeutic tool in gynecology.

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