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Factors, including transforming growth factor β, released in the glioblastoma residual cavity, impair activity of adherent lymphokine-activated killer cells

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Abstract. Adherent lymphokine-activated killer (A-LAK) cells were obtained from peripheral blood lymphocytes of patients with recurrent glioblastoma. In vitro features of A-LAK cultures were assessed in comparison to those of non-adherent lymphokine-activated killer (NA-LAK) cells of the same patients with regard to cytotoxic activity, proliferation and surface markers. Only in a minority of cases did A-LAK cells show a markedly higher cytotoxicity on K562, Daudi and allogeneic glioblastoma cells. Nevertheless, A-LAK cells proliferated significantly better than NA-LAK and contained higher percentages of $CD16^+$, $CD56^+$ and $CD25^+$ cells, indicating that A-LAK cells from these patients represent a subpopulation of lymphocytes enriched for activated natural killer cells. We also investigated whether immunosuppressive factor(s) were present in the tumour bed of recurrent gliomas. To this end, samples of glioblastoma cavity fluid (GCF), which accumulates in the cavity of subtotally removed tumour, were recovered and tested for the presence of immunosuppressive activity. All GCF samples analysed were shown to inhibit in vitro proliferation and antitumour cytotoxicity of 1-week-cultured A-LAK cells in a dose-dependent manner. Such GCF activity was effectively antagonized by a transforming growth factor β (TGFβ) neutralizing antibody, indicating the involvement of TGFβ in lymphocyte inhibition. These results show that in the tumour cavity remaining after subtotal glioblastoma resection a marked immunosuppressive activity, probably due to local release of TGFβ, is present; such activity may negatively influence the therapeutic effectiveness of local cellular immunotherapy.

Key words: Glioblastoma – A-LAK cells – In vivo immunosuppression – TGFβ

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Introduction

Glioblastomas are tumours for which no curative treatment regimen exists. Conventional therapy for these high-grade gliomas consists of surgical debulking followed by radiation therapy and/or chemotherapy. Although innovative forms of radiotherapy and new drug combinations have produced a longer survival time [36], the prognosis for these patients remains poor.

Even though glioma patients show an impaired cell-mediated immunity [2, 17], their survival apparently correlates with the degree of lymphocytic infiltration in the tumour [22]. These findings have stimulated interest in the immunobiology of brain tumours with the aim of evaluating whether immunological treatments could represent a new therapeutic modality for these neoplasias. Immunotherapeutic approaches have included the use of non-specific immunostimulants [12], and activated lymphocytes in combination with interleukin-2 (IL-2) [19] or conjugated with bispecific hybrid antibodies [21] injected directly into the tumour bed of recurrent gliomas. In fact, for tumours confined to a certain body compartment, i.e. the central nervous system for malignant astrocytomas, a local administration of cells and lymphokines may prove more effective than a systemic approach. In particular lymphokine-activated killer (LAK) cells have been used for the local treatment of gliomas [19]; although sustained clinical responses have been claimed, little advance has been made in terms of patient survival [19].

The poor therapeutic benefit obtained by such immunotherapeutic studies is likely also to be due, among other factors, to an immunoinhibitory activity mediated by gliomas [27]. In fact, malignant glial tumours are known to release immunosuppressive factors such as prostaglandin E_2 (PGE_2) [29] and transforming growth factor β (TGFβ) [1, 3]. The latter has been shown to inhibit the mitogenic actions of IL-2 on T lymphocytes [6, 13], suppress natural killer (NK) cell function [26] and LAK cell generation from peripheral blood lymphocytes of healthy donors [14, 20].

In 1989 we started a clinical trial of local adoptive immunotherapy with IL-2 plus adherent LAK (A-LAK) cells for the treatment of recurrent glioblastoma. A-LAK cells, a subpopulation of lymphoid cells purified by adherence to plastic, can exert a stronger antitumour activity in vitro than can non-adherent LAK (NA-LAK) cells [18]. Moreover, A-LAK cells can be isolated and expanded also from the blood of patients with brain tumours [37] thus allowing the therapeutic potential of these cells to be tested in glioma-bearing patients. In the context of this trial we investigated whether immunosuppressive factors were present at the tumour site where they could inhibit the activity of transferred lymphocytes, thereby influencing the outcome of treatment. Data reported here indicate that a marked immunoinhibitory activity is present in the tumour bed cavity and that the bulk of such activity may be ascribed to TGFβ.

Materials and methods

Patients and treatment schedule. Nine patients with recurrent glioblastoma were treated with local immunotherapy with A-LAK cells and IL-2 between 1989 and 1991. All patients were submitted to subtotal tumour excision followed by chemotherapy and radiotherapy. Eligible patients had not received any antitumour therapy for at least 3 months before entering the study. Steroid medication was discontinued at least 4 days before blood was donated for the generation of A-LAK cells. The treatment schedule consisted of daily injections of recombinant IL-2 (6×10^5 IU/day) for 5 days each week for 2 weeks while A-LAK cells (1.5×10^8) were administered on day 1 of each week of treatment. The infusion of IL-2 and cells directly in the cavity of subtotally resected tumour and the withdrawal of glioblastoma cavity fluid (GCF) samples was performed through an Ommaya reservoir placed into the resection cavity at the time of surgery.

Generation of A-LAK cells. Peripheral blood mononuclear cells (PBMC) were separated from buffy coats of venous blood of glioma patients or healthy donors by Ficoll/Hypaque gradient centrifugation. Cells collected at the gradient interface were washed three times with phosphate-buffered saline PBS, resuspended in complete medium consisting of RPMI-1640 (MA Bioproducts, Walkersville, Md., USA) supplemented with 5% pooled human heat-inactivated serum, 2 mM glutamine, 20 mM HEPES buffer, 100 units/ml penicillin G and 100 μg/ml streptomycin. PBMC were depleted of monocytes and B cells by a double incubation in complete medium in a plastic flask in a horizontal position for 1 h. Cells that did not adhere to plastic were resuspended in complete medium supplemented with 6000 IU/ml IL-2 (Proleukin, kindly provided by EuroCetus BV, Amsterdam, The Netherlands) at a concentration of 2×10^6 cells/ml and placed in culture flasks (Corning, Corning, N. Y., USA). The flasks were incubated in a horizontal position at 37° C in an atmosphere of 5% CO_2 in air for 48 h. At that time the floating cells (NA-LAK) were decanted from the flasks while cells adhering to the plastic (A-LAK) were washed three times with complete medium to remove cells that were not firmly attached to plastic and then cultured in complete medium in the presence of 6000 IU/ml IL-2. Simultaneously the recovered NA-LAK cells were cultured separately under the same growth conditions.

Reagents. GCF samples were recovered from the cystic cavity remaining after tumour excision of glioblastoma patients before immunotherapy. GCF samples from glioblastoma patients who did not enter the clinical study were also used. The samples were centrifuged, filtered through 0.22-μm filters (Millipore S. A., Molsheim, France), heat-inactivated (56° C, 30 min) and stored at –20° C until assayed. Human recombinant TGFβ1 was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Human recombinant tumour necrosis factor α (TNFα) was obtained from EuroCetus. The polyclonal neutralizing antibody to TGFβ (anti-TGFβ) was purchased from British Bio-technology Ltd. (Cowley, Oxford, UK). The unrelated control monoclonal antibody (mAb) MAA-R24 (anti-GD3) was a generous gift of Dr. A. N. Houghton (New York).

Analysis of TGFβ production. In order to assess the ability of glioblastoma cells to produce TGFβ, an intracellular indirect immunofluorescence assay was performed on the tumour cell line established from patient 01 according to a technique described by Sander et al. [28]. The anti-TGFβ used was purchased from British Bio-technology Ltd.; a swine anti-(rabbit Ig) fluorescein-conjugated antibody (Dako, Glostrup, Denmark) was utilized as secondary antibody. For the same purpose fresh tumour cells from patient 07 were analysed by immunocytochemistry with the above antibody and a biotin-conjugated swine anti-(rabbit Ig) antibody (dilution 1 : 200; Vector, Burlingame, Calif.) using a technique described in detail elsewhere [24].

Proliferation. In order to assess the proliferative activity of A-LAK cells after 7 days of culture before in vivo injection, lymphocytes (10^5 cells/well) were seeded in 96-well round-bottomed plates (Costar, Cambridge, Mass., USA) in complete medium without IL-2, pulsed with tritiated thymidine ([^{3}H]dT) (NEN, Florence, Italy) (1 μCi/well) and incubated for 6 h in a humidified atmosphere of 5% CO_2 in air. A Skatron harvester was used to harvest the cells and radioactivity was counted by using a beta scintillation counter. To evaluate the immunosuppressive property of GCF on A-LAK cells and its possible reversal by cytokines, various concentrations of human heat-inactivated serum (control) or increasing dilutions of GCF were added to lymphocytes in the presence of 600 IU/ml of IL-2. In some experiments various doses of TNFα (125–500 U/ml) were added to the plates. Plates were incubated at 37° C in an atmosphere of 5% CO_2 in air for 24 h. [^{3}H]dT incorporation was determined during the last 6 h of culture. To assess the possible presence of TGFβ in GCF samples, the samples were preincubated for 1 h at 4° C with various dilutions of anti-TGFβ. At that time A-LAK cells were added to the plates and the experiments performed as described above.

Cytotoxic assay. Target cells were incubated with 0.2 ml ^{51}Cr (Amersham, Buckingam, UK) for 1.5 h at 37° C and then washed three times with complete medium. A total volume of 200 μl complete medium containing 10^3 target cells and various numbers of effector cells at final effector:target (E:T) ratios of 100, 50, 25 and 12:1 was placed in 96-well round-bottomed plates, centrifuged and incubated at 37° C in a humidified atmosphere of 5% CO_2 in air. After 4 h an aliquot (100 μl) of supernatant was collected and counted in a beta scintillation counter. The percentage ^{51}Cr release was calculated according to the following formula:

$$(E–S)/(M–S) \times 100$$

where E is the mean ^{51}Cr release (cpm) in the presence of effector cells, S is the mean ^{51}Cr spontaneously released by the target cells incubated with complete medium alone and M is the mean maximum release obtained by incubating target cells with 100 μl 1% NP-40 detergent. The human erythroleukemia K562, the Daudi lymphoma line and a glioblastoma line established from patient 01 before immunotherapy were used as target cells. In some experiments effector cells were preincubated with various concentrations of GCF from glioma patients or increasing doses of TGFβ. After 18 h target cells were added to the plates and the experiment was performed as described above.

Indirect immunofluorescence analysis. Lymphocytes (2×10^5) were suspended in 0.1 ml/well in 96-well flat-bottomed plates (Costar), incubated at 4° C for 30 min with the primary mAb, washed three times with PBS and further incubated at 4° C for 30 min with 0.1 ml 1:30 diluted fluorescinated goat anti-(mouse Ig) (Technogenetics, Trezzano s/N, Italy). After three washes with PBS, the stained cells were mechanically removed and all samples analysed by a fluorescence-activated cell sorter (EPICS C, Coulter Corporation, Hialeah, Fla., USA). Background fluorescence (determined by control mAb staining) was subtracted in each case. The following mAb were used: OKT3 (Ortho, Raritan, N. J.,

Table 1. Cytotoxic activity of adherent and non-adherent lymphokine-activated killer cells (A-LAK and NA-LAK cells) from patients with glioblastoma[a]

Patient no.	Lysis (%) of					
	K562		Daudi		Allo-GBL[b]	
	A-LAK	NA-LAK	A-LAK	NA-LAK	A-LAK	NA-LAK
01	67[c]	20	38	7	ND[d]	ND
02	98	64	78	70	69	49
03	95	85	84	81	100	84
04	68	66	80	68	65	49
05	78	75	87	72	15	42
06	75	78	80	70	56	45
07	75	78	71	66	88	56
08	64	63[e]	73	35[e]	73	22[e]
09	54	59	88	85	100	92
Mean ± SD	75 ± 14	65 ± 19	75 ± 15	62 ± 25	71 ± 28	55 ± 23

[a] A-LAK and NA-LAK cells were tested after 7 days of in vitro culture with interleukin-2 (IL-2)
[b] Allogeneic glioblastoma cell line (established from patient 01 before immunotherapy)
[c] Data refer to the effector: target ratio of 50 : 1
[d] ND, not done
[e] NA-LAK cells were tested after 12 days of in vitro culture with IL-2

Table 2. Proliferative activity of A-LAK and NA-LAK cells from patients with glioblastoma[a]

Patient no.	[^{3}H]dT incorporation (cpm)	
	A-LAK	NA-LAK
01	42447	1851
02	36833	20759
03	62118	24220
04	166519	26936
05	33583	46237
06	69544	86893
07	80480	15694
08	69176	19473[b]
09	80812	40090
Mean ± SD	71279 ± 39963	31350 ± 24550

[a] A-LAK and NA-LAK cells were tested in a 6-h proliferative assay after 7 days of in vitro culture with IL-2
[b] NA-LAK cells were tested after 12 days of in vitro culture with IL-2

USA), anti-Leu11b (CD16), anti-Leu19 (CD56), anti-IL-2 receptor (p55) (CD25) (Becton Dickinson, Calif., USA).

Cell viability. A-LAK cell viability was determined by trypan blue exclusion after 24 h of incubation at 37° C in a humidified atmosphere of 5% CO_2 in air in the presence of either human heat-inactivated serum from normal donors (dilution 1 : 8) or GCF from glioma patients (dilution 1 : 8).

Statistics. Statistical analysis used Student's *t*-test.

Results

Functional evaluation of A-LAK cells from glioma patients before in vivo injection

Cytotoxic activity of A-LAK in comparison to that of NA-LAK cells from the same patients was evaluated after 7 days of culture with 6000 IU/ml IL-2. The experiments included as targets the tumour lines Daudi and K562, to evaluate LAK and NK activity respectively, and one glioblastoma cell line established from patient 01 before immunotherapy. As shown in Table 1, A-LAK cells were markedly more lytic than NA-LAK on K562 in patients 01 and 02 and on Daudi in patients 01 and 08; in all but one patient (05) A-LAK cells lysed glioblastoma targets better than NA-LAK. The mean percentage of lysis on each target for A-LAK and NA-LAK cells was 75 ± 14 (mean ± standard deviation) versus 65 ± 19 on K562, 75 ± 15 versus 62 ± 25 on Daudi and 71 ± 28 versus 55 ± 23 on glioblastoma cells respectively. These differences, however, were not statistically significant.

The proliferative activity of A-LAK cells as evaluated after 7 days of culture was higher than that of NA-LAK in all but two patients (05 and 06) (Table 2). In fact, the mean [^{3}H]dT incorporation (cpm) was 71279 ± 39963 (mean ± SD) for A-LAK versus 31350 ± 24550 for NA-LAK cells (Table 2). This difference was statistically significant ($P < 0.05$) and suggested that A-LAK cells from these patients represent a population enriched for lymphocytes with high proliferative activity.

Finally, the expression of the immunological markers CD3 (T lymphocytes), CD16 (NK cells), CD56 (NK, activated lymphocytes) and CD25 (α chain of IL-2 receptor, p55) by A-LAK was studied and compared to that of NA-LAK cells from the same patients after 7 days of culture. Results of Table 3 show that in all but one case (07) A-LAK cultures contained less than 60% of CD3$^+$ cells (mean ± SD: 41 ± 21%), which represented up to 91% of lymphocytes in the NA-LAK population (mean ± SD: 62 ± 19%); this difference, however, was not statistically significant. Percentages of both CD16$^+$ and CD56$^+$ cells were significantly higher in A-LAK (mean ± SD: 41 ± 16 and 48 ± 18) than in NA-LAK cultures (mean ± SD: 18 ± 9 and 17 ± 9) ($P < 0.05$ in both cases). Finally, the proportion of lymphocytes positive for CD25 expression was found to be greater in A-LAK than in their NA-LAK counterparts (mean ± SD: 43 ± 22% and 19 ± 12%

Table 3. Surface phenotype of A-LAK and NA-LAK cells from a group of patients with glioblastoma[a]

Patient no.	Lymphocytes (%) positive for[b]							
	CD3		CD16		CD56		CD25	
	A-LAK	NA-LAK	A-LAK	NA-LAK	A-LAK	NA-LAK	A-LAK	NA-LAK
03	34	67	58	10	36	8	15	4
04	26	67	58	25	66	21	44	43
05	9	63	47	15	75	16	57	21
06	49	33	43	29	35	30	27	23
07	75	91	40	7	59	6	75	19
08	54	41[c]	17	28[c]	36	27[c]	57	10[c]
09	37	69	22	10	28	9	24	12
Mean ± SD	41 ± 21	62 ± 19	41 ± 16	18 ± 9	48 ± 18	17 ± 9	43 ± 22	19 ± 12

[a] A-LAK and NA-LAK cells were tested after 7 days of in vitro culture with IL-2

[b] The expression of the different markers was evaluated by indirect immunofluorescence

[c] NA-LAK cells were tested after 12 days of in vitro culture with IL-2

Table 4. Inhibition of A-LAK cell proliferation by glioblastoma cavity fluid (GCF) from glioblastoma patients

Dilution of GCF	Inhibition (%) by GCF from patient[a]									
	01	02	03	07	09	C. E.	B. L.	T. C.	S. A.	F. L.
1 : 2	92[b]	66	73	60	ND[c]	ND	ND	ND	99	99
1 : 4	42	68	68	53	77	96	46	81	98	96
1 : 8	37	48	61	22	74	96	43	56	92	90
1 : 16	28	25	53	7	42	86	19	34	87	86
1 : 32	13	8	30	5	8	76	13	30	84	71
1 : 64	5	–	6	–	13	63	15	18	79	64

[a] A-LAK cell cultures of five patients who entered the clinical study (01 – 09) were tested with autologous GCF after 1 week of IL-2 exposure (left panel); A-LAK cells from a healthy donor were tested, after 1 week of IL-2 exposure, with GCF obtained from five patients who did not enter the clinical study (right panel)

[b] Data are given as percentage inhibition of A-LAK cell proliferation calculated from control ([^{3}H]dT incorporation which ranged between 43 376 cpm and 219 774 cpm)

[c] ND, not done

respectively) ($P < 0.05$). These data indicate that A-LAK cells contain a higher percentage of activated NK cells.

Inhibition of A-LAK cell proliferation by GCF. Role of TGFβ

During the clinical trial of local adoptive immunotherapy with IL-2 plus A-LAK cells we calculated that the treatment schedule adopted ensured concentrations of IL-2 at the tumour site that are sufficient to maintain A-LAK activity in vitro (Boiardi et al., manuscript in preparation). Because of the weak clinical response obtained in these patients, we decided to investigate whether immunosuppressive factors were present in the resection cavity where A-LAK cells and IL-2 were injected. To this end, samples of GCF recovered from patients before immunotherapy were tested for inhibitory activity on A-LAK cell proliferation. The limited number of patients eligible for GCF drawing and the reduced amount of GCF samples from each patient limited the number of experiments; therefore, GCF samples from glioblastoma patients who did not enter the clinical trial were also used. Proliferation assays revealed that GCF can markedly reduce, in a dose-dependent manner, proliferation of 1-week-cultured A-LAK cells even in the presence of 600 IU/ml IL-2 (Table 4). Patients' GCF samples showed dose-dependent inhibitory activity both on autologous A-LAK and on A-LAK cells from a healthy volunteer (Table 4, left and right panels respectively). As a control, human serum of normal donors added to A-LAK cultures showed no inhibitory activity (data not shown). The suppressive effect of GCF on A-LAK cell growth could not be explained by a direct cytotoxic effect of GCF on lymphocytes since their viability was greater than 85% after 24 h of incubation with GCF (dilution 1 : 8) (data not shown).

Since previous studies have demonstrated that glioblastoma cells in culture secrete potent inhibitors of lymphocyte activation, in particular TGFβ [1], experiments were conducted to examine whether this cytokine, potentially present in GCF, could be responsible for the suppressive activity observed on A-LAK cell proliferation. The results of a representative experiment are reported in Table 5. The addition of anti-TGFβ but not of an anti-GD3 (MAA-R24) unrelated control mAb (data not shown) was able to counteract, in a dose-dependent fashion, the inhibitory effect of GCF from patient 07 on autologous, 1-week-cultured A-LAK cell proliferation. Similar findings were ob-

Table 5. Inhibition of autologous A-LAK cell proliferation by glioblastoma cavity fluid (GCF) and by supernatant from the autologous glioblastoma cell line (patient 07); reversal by anti-transforming growth factor β antibody (anti-TGFβ)

Incubation with	Dilution (final volume)	Concentration of Ab (μg/ml)	[^{3}H]dT[a] (cpm)	Inhibition[b] (%)
CM			137291	–
GCF	1:2		55223	60
	1:4		65148	53
	1:8		106663	22
	1:16		128260	7
	1:32		130573	5
	1:64		138263	–
GCF+ anti-TGFβ	1:2	60	129926	5
	1:2	40	95571	30
	1:2	20	84600	38
	1:2	10	70539	49
	1:2	5	55513	60
	1:2	2.5	52216	62
GBLsup[c]	1:2		89288	35
	1:4		89774	35
	1:8		92337	33
	1:16		122526	11
	1:32		128393	6
	1:64		133091	3
GBLsup+ anti-TGFβ	1:2	60	154160	–
	1:2	40	134948	2
	1:2	20	124168	10
	1:2	10	107990	21
	1:2	5	106385	23
	1:2	2.5	93695	32

[a] Proliferative activity of A-LAK cells was evaluated in a 24-h [^{3}H]dT incorporation assay after 1 week of culture with IL-2

[b] Percentage inhibition was calculated in comparison with control, represented by A-LAK cells incubated in complete medium (CM)

[c] Supernatant of cultured autologous glioblastoma cells

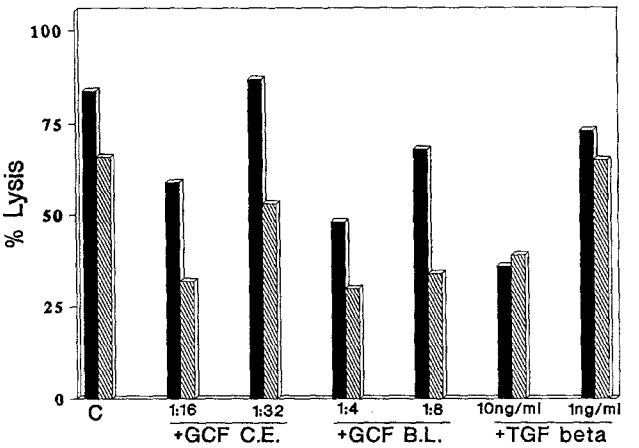

Fig. 1. Inhibition of allogeneic A-LAK cell cytotoxicity by GCF from glioblastoma patients. A-LAK cells from a glioblastoma patient (S. M.) were tested, after 1 week of culture with interleukin-2 (IL-2), with GCF samples from two other patients (C. E. and B. L.) in the presence of 600 IU/ml IL-2. Control (*C*) was represented by preincubation of A-LAK cells with human heat-inactivated serum. Data are given as mean values of cytotoxicity as determined in two independent experiments. Data refer to an effector: target ratio of 50:1. Spontaneous release from each target cell was recalculated after 4 h incubation with GCF at the higher concentration used. Targets: *black columns*, K562; *hatched columns*, allogeneic glioblastoma cell line

tained with GCF from patients 03 and 09 although in the case of patient 09 no complete reversal was achieved, suggesting that immunosuppressive factors other than TGFβ could also be present in GCF. However, it is noteworthy that in these experiments a high concentration of anti-TGFβ (i.e. 60 μg/ml) was required to antagonize the immunosuppressive effect of GCF completely, thus suggesting the presence of large amounts of TGFβ in the tumour bed cavity. As a positive control, TGFβ (0.1–20 ng/ml) was shown to partially inhibit the proliferative activity of A-LAK cultures in a dose-dependent manner (data not shown).

The production of TGFβ was confirmed in patients 01 and 07 by using indirect immunofluorescence and immunocytochemistry respectively (data not shown). Moreover, supernatant of cultured glioblastoma cells from patient 07 was found to inhibit autologous A-LAK cell proliferation in a dose-dependent manner and to be effectively counteracted by the addition of escalating concentrations of anti-TGFβ (Table 5).

Since TNFα has been found to neutralize TGFβ immunosuppressive activity at least partially [5, 8, 23, 33], experiments were performed to examine whether addition of this cytokine could restore a normal proliferation of 1-week-cultured A-LAK cells. We found that TNFα at concentrations ranging from 125 U/ml to 500 U/ml has no neutralizing activity on GCF-induced inhibition of A-LAK cell proliferation even in the presence of 600 IU/ml IL-2 (data not shown).

Inhibition of A-LAK cell cytotoxic activity by GCF

The treatment schedule adopted in the clinical study consisted of A-LAK cell administration on day 1 of each week of treatment and daily injections of IL-2 for 4 more days after cell administration. We investigated, therefore, whether lymphocytes could retain antitumour activity in the microenvironment of the glioma cavity since, according to the above data, their proliferative potential may be markedly reduced at the tumour site even in the presence of adequate concentrations of IL-2. To this end we tested 1-week-cultured A-LAK cells from a glioblastoma patient, who did not participate in the clinical study, for cytotoxic activity after 18 h incubation in the presence of increasing concentrations of allogeneic GCF and 600 IU/ml IL-2. The limited availability of fluid samples allowed the GCF of only two patients to be assayed; in both cases cytotoxicity of A-LAK cells was found to be reduced by GCF in a dose-dependent manner (Fig. 1). Since the possible role of TGFβ in this phenomenon could not be more directly tested by blocking experiments with neutralizing antibody, serial concentrations of TGFβ were used and shown to be able to inhibit A-LAK cell cytotoxicity (Fig. 1). Similar results were obtained with A-LAK cells from a healthy volunteer exposed to the same GCF samples (data not

shown). These results indicate that lymphocytes, when injected into the tumour bed cavity, meet with unfavourable local conditions that could reduce their tumour-killing effectiveness.

Discussion

In the present paper we show that only in a minority of cases do A-LAK cells generated from the blood of patients with glioblastoma have higher levels of cytotoxicity against K562, Daudi and glioblastoma cell targets in comparison with NA-LAK cells from the same patients. Nevertheless, A-LAK cells proliferated better than NA-LAK and this fact is of interest from a therapeutic point of view, because lymphocytes could expand in vivo under the influence of IL-2 and maintain their antitumour activity as they multiply. As for the immunophenotype, greater percentages of $CD56^+$, $CD16^+$ and $CD25^+$ cells and a smaller proportion of $CD3^+$ cells were observed in A-LAK cultures as compared with their NA-LAK counterparts, indicating that A-LAK population is enriched for activated NK cells. Altogether our preclinical data are in agreement with previous findings [37]. Despite these interesting in vitro features of A-LAK cells, no clinical data have been published confirming the results obtained in animal models [31] showing a higher therapeutic potential of such effector cells as compared to standard LAK cells. Our clinical results (Boiardi et al., manuscript in preparation) do not differ from those reported in the literature (for review see [19]). The reasons for such a failure are unknown but it could be hypothesized that in vivo biological factors exist that may reduce the antitumour effectiveness of A-LAK cells plus IL-2 immunotherapy in glioma patients.

Several reports have already shown that glioma-bearing patients present with impaired immune functions [2, 17]. Of note are recent studies that correlate these immunological dysfunctions with the production of immunosuppressive mediators by gliomas [27]. In fact, these neoplasms have been shown in vitro to produce factor(s), including TGFβ, that inhibit a variety of lymphocyte functions [1, 3, 4, 6, 14, 15, 16, 29, 32, 33]. Whatever its source, TGFβ can inhibit IL-2-induced T cell proliferation [6, 13], cytotoxic T cell generation [7, 20, 23], production of tumour-infiltrating lymphocytes (TIL) [25] and the generation of LAK cells [14, 20]. Recently, a highly immunogenic mouse tumour transfected with a TGFβ gene has been shown to escape the host immune defence in vivo [35].

On the basis of these findings we investigated whether local conditions at the tumour site could reduce the effectiveness of adoptively transferred A-LAK cells and whether this impairment could be mediated by TGFβ. For this purpose, samples of GCF that accumulate in the cystic cavity remaining after tumour excision were recovered through the Ommaya reservoir and tested for the presence of immunosuppressive activity. This GCF was found in vitro to inhibit A-LAK cell proliferation markedly and antitumour cytotoxicity in a dose-dependent manner even in the presence of 600 IU/ml IL-2, thus suggesting that injected lymphocytes may be prevented from proliferating and killing malignant cells by soluble factor(s) released at the tumour site. Although multiple mechanisms could explain this phenomenon, such as the presence of necrosis in the tumour bed cavity or the production of non-specific immunosuppressive mediators like PGE_2 [16], previous studies demonstrating that malignant gliomas can secrete TGFβ [1] and that lymphocytes possess large numbers of high-affinity receptors for TGFβ [26] prompted us to investigate whether TGFβ was present in GCF. We found that the addition of increasing concentrations of anti-TGFβ reduced the GCF-induced inhibition of A-LAK cell proliferation in a dose-dependent manner, and, at a concentration of 60 μg/ml, restored a normal proliferative rate in two out of three cases analysed. These data suggest that TGFβ produced in vivo by glioblastoma cells and present in GCF is likely to be a major factor responsible for reduced lymphocyte proliferation in vivo. The molecular basis of reduced lymphocyte proliferation in the presence of GCF remains to be determined although it has been proposed that an impaired expression of high-affinity IL-2 receptor may be involved [4]. Concerning the inhibitory effect of GCF on A-LAK cell cytotoxic activity, a possible role of TGFβ in reducing the efficacy of the lytic machinery of killer cells is suggested by experiments that showed that TGFβ can affect the pore-forming protein gene expression in human lymphocytes [34]. Unfortunately, the limited availability of GCF samples did not allow us to evaluate these hypotheses. It should be noted, however, that, with the exception of a report by Kuppner and colleagues [15], TGFβ is known to exert its inhibitory effect on LAK and T cell function in the early steps of lymphocyte activation [5, 7, 8, 14, 23] rather than in established A-LAK cell cultures as occurs in our system. Although TGFβ can inhibit A-LAK cell activity in a dose-dependent fashion, the level of such inhibition was always lower than that obtained with GCF. This effect could be due either to a higher concentration of TGFβ or to the presence of additional lymphocyte suppressor factor(s) in the GCF samples used in our experiments.

In order to restore a normal A-LAK cell function in the tumour cavity, one can hypothesize that the use of TGFβ-neutralizing antibodies could improve the therapeutic efficacy of killer cells. However, given the obstacles still inherent in antibody use in vivo [30], we wanted to see whether TNFα could counteract the GCF-induced inhibition of A-LAK cell proliferation, since TNFα had originally been described to antagonize the TGFβ-induced inhibition of cytotoxic T lymphocyte generation in mixed lymphocyte culture [23]. Similar data on the restoring activity of TNFα were subsequently obtained by other authors in different experimental systems [5, 8, 33]. We found that at doses ranging from 125 U/ml to 500 U/ml the addition of TNFα failed to increase the in vitro A-LAK cell proliferation. The reason for choosing such a dose range was that higher doses of TNFα are unlikely to be obtained in the glioma cavity without severe toxic effects. The possibility cannot be excluded, however, that higher doses of TNFα could have been effective in counteracting GCF immunosuppressive activity in vitro. Interestingly, it has recently been shown that tumour cyst fluid from 20 patients with high-grade gliomas is able to inhibit cytolytic activity of TNF on a TNF-sensitive tumour line. This was due to the

presence in tumour cyst fluid of soluble receptors for TNF shed by tumour cells, which act as blocking factors for this cytokine [9]. Finally, it must be noted that GCF samples from glioblastoma patients could contain different factors with immunomodulatory activity, which were not studied in our experiments.

It should also be stressed that the antitumour activity in vivo of adoptively transferred lymphocytes depends also on other factors including the capacity of killer cells to infiltrate the tumour mass. Experiments conducted in the model of multicellular spheroids grown from human glioma cell lines, which represent three-dimensional tumour tissue, showed that the capacity of LAK cells to infiltrate is limited [10]. Our clinical observations (Boiardi et al., manuscript in preparation) have revealed that tumour growth is usually localized in the deeper layers of the brain parenchyma surrounding the cavity of subtotally resected tumour, suggesting that even if an antitumour activity could be mediated in vivo by injected A-LAK cells, it would be limited to the superficial layers of the tumour tissue. One can speculate that the capacity of activated lymphocytes to infiltrate, even when enriched for $CD56^+$ cells which have recently been shown to migrate more readily than others into glioma spheroids [11], could be influenced by the presence of immunosuppressive factor(s) in GCF.

In the light of these data we propose that the poor clinical responses observed during local adoptive immunotherapy could be due to the presence at the tumour site of potent, tumour-secreted inhibitors of lymphocyte function. Given the diffusely infiltrative nature of malignant gliomas, the relatively slow tumour infiltration of killer cells and the inhibition of lymphocyte proliferation and cytotoxicity by GCF, it is unlikely that intralesional activated lymphocytes plus IL-2 therapy can provide effective tumoricidal activity. Such an immunotherapy, however, may become more effective when the immunosuppressive activity exerted by the glioma cells is successfully counteracted.

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