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Generation of lymphokine-activated killer cells in human ovarian carcinoma ascitic fluid: identification of transforming growth factor-β as a suppressive factor*

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Summary. The effect of cell-free ascitic fluid from patients with epithelial ovarian carcinoma on the generation of lymphokine-activated killer cells (LAK) was compared to the activity generated in control medium containing 10% fetal bovine serum, using Daudi target cells. Samples of ascitic fluid from nine different patients tested inhibited LAK generation. Suppressive activity was evident as early as 24 h of incubation in the presence of ascitic fluid and increasing suppression developed with prolonged exposure. Suppression was concentration-dependent, present at 10%–20% and increasing with concentrations up to 80%. The suppressive effect of ascitic fluid was only partially reversed on increasing the concentration of interleukin-2 (IL-2) from 10 units to 1000 units/ml. Activated LAK appeared to maintain the majority of their activity on further culture in ascitic fluid in the presence of IL-2 but further enhancement of lytic activity was prevented. Fractionation of a suppressive sample by HPLC, using 0.1 M KCl/acetic acid buffer pH 2.6, revealed that the dominant peak of suppressive activity eluted at 25 kDa; with pH 7.0 TRIS-buffered saline, most of the activity was lost on the column. Antibody neutralization studies of the 25-kDa suppressive peak as well as on whole ascitic fluid have revealed that transforming growth factor β (TGFβ) is the major suppressive factor present in ascitic fluid. Factors that suppress LAK generation in vitro were present in all samples tested. The effect on the lytic activity of activated LAK cells was minimal. This suggests that, in the clinical setting, the greatest impact would be achieved by activating LAK cells ex vivo and subsequently transferring them to the peritoneal cavity in the presence of IL-2 rather than by attempting to generate them in situ by injecting IL-2 into the peritoneal cavity. However, reversal of TGFβ-mediated suppression in situ may be necessary to allow local proliferation of LAK cells to achieve an effective killer-to-target ratio.

Introduction

Lymphokine-activated killer (LAK) cells have the ability to lyse a broad range of both autologous and allogeneic targets in vitro [10, 22], but their efficacy when used clinically appears much less than would have been predicted from in vitro and animal studies [6, 18, 23, 32, 33]. We have suggested that such inefficacy could be due to (a) LAK cells killing primarily the differentiated (non-stem cell) component of the tumor, thereby showing good activity in cytotoxic assays in vitro but little efficacy against stem cells or, (b) active suppression of lytic effectors by certain cell populations in the tumor. Products of these cells might also be active.

A number of immunosuppressive factors, both humoral and cellular, have been described in patients with advanced malignancy [3–5, 9, 12–14, 19, 20, 25, 34, 35, 39]. In this paper, we report on studies to test for suppression of LAK activation by cell-free ascitic fluid from patients with epithelial ovarian carcinoma and the characterization of the suppressive activity present.

Materials and methods

Procurement and preparation of ascitic fluid. Ascites samples were obtained at the time of routine diagnostic or therapeutic paracentesis of patients with known epithelial ovarian carcinoma attending the OCTRF-Hamilton Regional Cancer Center or the Henderson General Hospital, Hamilton, Ontario. All patients had advanced disease (FIGO stage III or IV) and had relapsed after initially receiving platinum-based chemotherapy. Samples were obtained at least 4 weeks after the last chemotherapy treatment. Fluid was collected in sterile 1-l vacuum bottles and kept at 4° C until processed (within 24 h of removal from the patient). The fluid was transferred to sterile 1-l centrifuge bottles and centrifuged at 500 *g* for 10 min. The supernatant fluid (ascitic fluid) was removed and stored

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at 4° C. Samples were centrifuged at 1000 *g* for 10 min prior to addition to LAK culture flasks.

Generation of LAK effector cells. Peripheral blood mononuclear cells (PBM) were isolated from peripheral blood by separating heparinized venous blood obtained from three normal donors (HH, VH and JB) on a Histopaque-1077 (Sigma Chemical Co., St. Louis, Mo) cushion. The PBM obtained were then cultured at a concentration of 2×10^6/ml in AIM-V serum-free medium (Gibco-BRL, Burlington, Ont., Canada) or RPMI-1640 medium, containing 10% fetal bovine serum (Gibco), 100 IU/ml penicillin G, 100 µg/ml streptomycin, 2 mM L-glutamine and 10 mM HEPES (R-10 medium), placed in 25-cm^2 tissue-culture flasks (Falcon, Oxnard, Calif.) containing 100 units/ml recombinant human interleukin-2 (IL-2) (Assoc. Biomed. Systems, Buffalo, NY) and incubated supine at 37° C in 5% CO_2. The effect of adherent cells on LAK generation was tested by allowing the PBM to incubate in the culture flask for 1 h and then transferring the non-adherent cells to a new 25-cm^2 culture flask for the remainder of the culture period and comparing the activity of these cells with PBM cultures where adherent cells were not removed. To some flasks, ascitic fluid (up to 80% v/v) or phosphate-buffered saline (PBS) 80% (v/v) was added to the culture medium. The effector cells were harvested after 4 days and subsequently used in cytolytic assays. Cells were counted using a hemocytometer and viability was assessed by trypan blue exclusion. To determine the effect of length of culture in ascitic fluid on LAK activity, PBM were cultured in R-10 medium containing 100 U/ml IL-2 and were transferred to ascitic fluid (80% in R-10+100 units/ml IL-2) after 1,2 or 3 days or were cultured in 80% ascitic fluid or R-10 for 4 days and then harvested. The effect of IL-2 concentration was tested by culturing PBM in R-10 or 80% ascitic fluid containing 10, 100 or 1000 units/ml IL-2 for 4 days. To test the effect of ascitic fluid on activated LAK cells, PBM were generated in R-10+100 units IL-2/ml for 4 days and were subsequently cultured in R-10+100 units IL-2/ml or 80% ascitic fluid in R-10+100 units IL-2/ml for an additional 4 days.

^{51}Cr-release assay. Daudi target cells (obtained from ATCC, Rockville, Md.) were maintained in suspension culture in R-10 medium. Daudi cells in log-phase growth were harvested by centrifugation, incubated for 1.5–2 h with 200 µCi/ml sodium [^{51}Cr]chromate and washed three times. The targets were then resuspended in RPMI-1640 medium with 10% heat-inactivated fetal bovine serum but otherwise containing the same additives as R-10 (R-10HI medium). A sample of 5000 target cells in 100 µl R-10HI was added to quadruplicate wells of round-bottom 96-well microtrays (Flow Laboratories Inc., McLean, Va.) to which was added either 100 µl R-10HI, 100 µl R-10HI containing effector test cells at a ratio of effector to target cells ranging from 25:1 to 3.125:1 or 100 µl NP-40. After 4 h incubation at 37° C/5% CO_2, a 100-µl aliquot of supernatant was removed and the percentage specific ^{51}Cr release (*P*) calculated from:

$$P = 100 \times \frac{(\text{release with effectors} - \text{release with medium})}{(\text{release with NP-40} - \text{release with medium})}$$

The mean and SEM of quadruplicates was determined. When the test effectors in the assay were serially two-fold diluted, the titration curve allowed calculation of the parameter $N\alpha t \times 10^3$ using a previously described computer program [7, 28], where *N* represents the number of effectors per well, *t* is the effector-target cell interaction time, and α is a constant proportional to the frequency of cytotoxic cells. The amount of activity that was required to lyse 20% of the target cells during the assay and that was equivalent to 223 $N\alpha t \times 10^3$ units was defined as 1 lytic unit (LU). No correlation was made for *t* and the data were expressed as LU ± 1 SEM.

High-performance liquid chromatography (HPLC) studies. A sample of ascites with suppressive activity (SCHM-1) was lyophilized to dryness by speed vacuum centrifugation and reconstituted to give a 5× concentrate. Neutral pH elution was performed by separating a 200 µl aliquot by HPLC on a TSK gel G3000SW column (Toyo Soda, USA Inc., Atlanta, Ga.) pre-equilibrated with TRIS (0.01 M) in sodium chloride buffer (0.1 M) at pH 7.0. Acid elution was performed by separating a 200 µl aliquot on a TSK gel G2000SW column pre-equilibrated with a 0.58% glacial acetic acid/KCl buffer (0.1 M) at pH 2.6. Fractions (5.0 ml) were eluted at a flow rate of 5 ml/min at room temperature. The column effluent was monitored at 280 nm using a Gilson 111B ultraviolet detector (Mandel Scientific Co. Ltd., Rockwood, Ont., Canada) connected to a Linear 1200 chart recorder. The columns were calibrated using the following markers: bovine serum albumin (63 kDa), cytochrome *c* (12.500 kDa), vitamin B_{12} (1.355 kDa) and phenol red (0.376 kDa).

Fractions were passed through a 0.22 µm Millex-GV filter. The fractions were then tested in 24-well plates (Nunclon-Intermed, Denmark) by culturing $(1-2) \times 10^6$ PBM (harvested as previously described) in 1 ml R-10 medium containing 100 units/ml IL-2 and 20% (v/v) HPLC fractions, and 20% unfractionated ascites or 20% TRIS buffer diluted in R-10 medium as controls for TRIS buffer elution fractions. For acid elution fractions, 1.0 ml HPLC fractions were diluted with 1.5 ml super-alkaline R-10 medium (100 ml R-10 medium to which was added 14 ml 3.25% sodium bicarbonate solution). They were subsequently tested in R-10 medium containing 100 units/ml IL-2 and 25% (v/v) diluted HPLC fractions (10% concentration of original HPLC fraction), with either 25% unfractionated ascites or 25% acid elution buffer diluted 1:1.5 with super-alkaline R-10 as controls. The plates were incubated at 37° C, 5% CO_2 for 4 days and then effectors were harvested. The activity of the effectors was tested at a 10:1 ratio against labelled Daudi targets in a ^{51}Cr-release assay as described in the preceding section.

Neutralization studies with anti-TGFβ antibody. Fractions from the acid HPLC elution studies with suppressive activity were tested by culturing PBM in 24-well plates as described above in the presence of R-10 medium containing 100 units IL-2/ml, and the ability of a neutralizing polyclonal rabbit IgG anti-TGFβ antibody (R&D Systems Inc., Minneapolis, Minn.) to reverse suppression was tested by adding 10 µg/ml antibody per well for 1 h prior to adding PBM. To assess for non-specific effects of non-immune rabbit IgG, PBM cultured in wells containing 10 µg/ml normal rabbit IgG were tested. To determine the effects of the neutralizing anti-TGFβ antibody on the suppressive activity of unseparated ascites fluid, PBM were cultured in wells containing 25% ascites fluid in R-10 and IL-2 at 100 units/ml, to which was added either control medium, anti-TGFβ antibody (10 µg/ml) or normal rabbit IgG (10 µg/ml).

Results

To determine the optimum conditions for LAK generation and subsequent detection of suppressive activity, PBM were grown in either AIM-V serum-free medium or R-10 medium. In addiditon, the activity of non-adherent cells was compared with the activity of unseparated PBM. The results of a representative experiment of three performed are shown in Table 1. Unseparated PBM showed significantly better activity when grown in R-10 medium than in AIM-V medium (24.6 ± 1.2 LU vs 3.8 ± 0.1 LU). Unseparated PBM consistently showed better activity than non-adherent PBM in both R-10 (24.6 ± 1.2 vs 8.2 ± 0.4 LU) and AIM-V medium (3.8 ± 0.1 vs 3.0 ± 0.1 LU).

To determine which of these media might be more sensitive in detecting suppressive activity of ascitic fluid, unseparated PBM were cultured in each medium containing a 50% concentration of ascitic fluid (either SCHM-1 or NAS-12) (Table 2). In AIM-V medium, NAS-12 ascitic fluid consistently enhanced LAK generation compared to AIM-V medium alone (10.1 ± 0.3 vs 3.8 ± 0.1 LU) suggesting the presence of factors that partially corrected the sub-optimal LAK growth conditions, whereas SCHM-1

Table 1. Effect of serum-free medium (AIM-V) and adherent cells on the generation of lymphokine-activated killer (LAK) activity

Effector	Medium	Lytic activity (LU ± 1 SEM)
Unseparated PBM	AIM-V	3.8 ± 0.1*
	R-10	24.6 ± 1.2
Non-adherent PBM	AIM-V	3.0 ± 0.1*, **
	R-10	8.2 ± 0.4*

* $P < 0.05$ compared to unseparated PBM cultured in R-10 by Student's *t*-test
** $P < 0.05$ compared to unseparated PBM cultured in AIM-V by Student's *t*-test

Table 2. Effect of AIM-V and R-10 medium on suppression of LAK generation by ascitic fluid

Medium	Ascitic fluid (50% v/v)	Lytic activity (LU ± 1 SEM)
AIM-V	–	3.8 ± 0.1
	NAS-12	10.1 ± 0.3*
	SCHM-1	0.7 ± 0.1*
R-10	–	24.6 ± 1.2
	NAS-12	4.1 ± 0.1**
	SCHM-1	1.6 ± 0.1**

* $P < 0.05$ compared to AIM-V medium alone by Student's *t*-test
** $P < 0.05$ compared to R-10 medium alone by Student's *t*-test

ascitic fluid showed inhibition (0.7 ± 0.1 vs 3.8 ± 0.1 LU). However, in R-10 medium both NAS-12 and SCHM-1 ascitic fluid showed suppressive activity when compared to R-10 medium alone (4.1 ± 0.1 and 1.6 ± 0.1 vs 24.6 ± 1.2 LU). Thus, further studies to characterize the suppressive activity of ascitic fluid were performed with unseparated PBM using R-10 medium.

To determine what proportion of patients with ovarian carcinoma produced ascitic fluid that could suppress the generation of LAK activity against Daudi target cells, PBM from a normal donor (VH) were grown in R-10 medium or 80% ascitic fluid in R-10 containing 100 U/ml IL-2. The data from two experiments testing ascitic fluid from seven additional patients are shown in Table 3. Ascitic fluid from all patients consistently showed significant suppression of LAK generation.

It was possible that the apparent suppressive effects of ascitic fluid in Table 3 were due to dilution of nutrients by the fluid. To test for this trivial explanation for the suppression, generation of LAK activity of several suppressive samples at 80% (v/v) in R-10 was compared to LAK generation with 80% PBS (v/v) in R-10 (Table 4). Although dilution of R-10 with 80% PBS resulted in a slight reduction of lytic activity (111.9 ± 4.2 LU for R-10 vs 91.2 ± 1.8 LU for 80% PBS), all ascitic fluid samples had a significantly greater reduction in lytic activity. Ascitic fluid did not appear to act via toxic effects on PBM as viability was uniformly high (>95%) as was cell recovery (>85%) (Table 4). To further exclude dilution of nutrients as an explanation for suppression, the dependence of suppression on ascitic fluid concentration was studied using two samples known to be suppressive (SCHM-1, NAS-12) (Table 5). SCHM-1 ascitic fluid continued to suppress

Table 3. Effect of ascitic fluid (80%) on the generation of LAK activity

Sample tested	Lytic activity (LU ± 1 SEM)	
	Expt. 1	Expt. 2
R-10	129.7 ± 2.4	107.0 ± 1.1
SCHM-1	17.8 ± 0.3*	27.2 ± 0.5*
HUG-2	12.4 ± 0.1*	8.5 ± 0.2*
MCL-1	12.0 ± 0.1*	27.4 ± 0.4*
PAS-1	90.8 ± 0.8*	57.9 ± 1.0*
LEP-2	83.2 ± 4.1*	84.6 ± 1.8*
NAS-12	25.0 ± 0.2*	80.2 ± 0.8*
KOT-1	65.1 ± 2.7*	42.0 ± 0.4*
FER-5	28.1 ± 0.4*	16.8 ± 0.6*
PHI-6	25.7 ± 0.6*	48.2 ± 0.8*

* $P < 0.05$ compared to R-10 by Student's *t*-test

Table 4. Effect of dilution of R-10 medium by 80% by phosphate-buffered saline (PBS) or ascitic fluid on lytic activity, cell recovery and viability

Sample tested	Lytic activity (LU ± 1 SEM)	Cell recovery compared to R-10 (%)	Viability (%)
R-10	111.9 ± 4.2	100	91.7
PBS	91.2 ± 1.8*	120	82.5
SCHM-1	21.7 ± 0.6**	115	96.9
FER-5	6.4 ± 0.1**	95	95.4
PHI-6	14.5 ± 0.2**	97	95.5
HUG-2	4.0 ± 0.2**	87	99.0

* $P < 0.05$ compared to R-10 medium by Student's *t*-test
** $P < 0.05$ compared to 80% PBS in R-10 by Student's *t*-test

Table 5. Effect of ascitic fluid concentration on generation of LAK activity

Sample concentration (%)	Lytic activity (LU ± 1 SEM)	
	SCHM-1	NAS-12
80	7.2 ± 0.1*	31.1 ± 0.8*
40	55.5 ± 1.0*	126.2 ± 4.8*
20	99.7 ± 2.9*	159.4 ± 1.5*
10	145.6 ± 7.4*	237.8 ± 9.7
0 (R-10)	179.7 ± 0.1	179.7 ± 0.1

* Significant suppression ($P < 0.05$ by Student's *t*-test) compared to R-10 control

LAK generation at concentrations as low as 10% while NAS-12 showed significant suppression at a 20% dilution.

To test whether increasing IL-2 concentration could overcome the suppressive effect of ascitic fluid, lytic activity of LAK generated in R-10 control medium or 80% HUG-2 ascitic fluid containing IL-2 at concentrations of 10, 100 or 1000 units/ml was determined (Table 6). At *all* concentrations of IL-2, LAK activity generated in ascitic fluid was significantly less than that in the control medium. Although increasing the concentration of IL-2 to 1000 units/ml allowed generation of more lytic activity compared to lower IL-2 concentrations, the suppressive effect of ascitic fluid was only partially reversed and LAK activity did not achieve the level obtainable with just 10 units IL-2 in medium alone.

Table 6. Effect of interleukin-2 (IL-2) concentration on generation of LAK activity in presence of ascitic fluid (AF)

IL-2 concentration (units/ml)	Lytic activity (LU ± 1 SEM)	
	R-10	80% HUG-2 AF (% control activity)
10	63.3 ± 4.5	5.5 ± 0.1* (8.7%)
100	103.9 ± 8.1	5.7 ± 0.2* (5.5%)
1000	148.3 ± 0.6	38.2 ± 1.2* (25.8%)

* $P < 0.05$ compared to R-10 medium control by Student's *t*-test

Table 7. Effect of ascitic fluid (AF) on activated LAK cells

Culture conditions	Lytic activity (LU ± 1 SEM)
R-10 × 4 days	39.5 ± 4.0
R-10 × 8 days	72.9 ± 2.2
SCHM-1 AF × 8 days	15.2 ± 0.8*
R-10 × 4 days → SCHM-1 AF × 4 days	30.0 ± 0.1*,**, ***
HUG-2 AF × 8 days	0.0*
R-10 × 4 days → HUG-2 AF × 4 days	34.6 ± 0.1*,**,***

* $P < 0.05$ compared to R-10 × 8 days by Student's *t*-test
** $P < 0.05$ compared to R-10 × 4 days by Student's *t*-test
*** $P < 0.05$ compared to AF × 8 days by Student's *t*-test

Some experiments on LAK generation were carried out with suppressive SCHM-1 ascitic fluid to determine when suppression occurred. PBM were cultured for 1, 2 or 3 days without ascitic fluid in R-10 containing 100 units IL-2/ml and then were transferred to SCHM-1 (80% v/v in R-10 medium +100 units IL-2/ml) for the remaining culture period, or were cultured in R-10 ± SCHM-1 for 4 days. Data from a representative experiment are shown in Fig. 1. Significant suppression occurred as expected when cells were cultured in 80% ascitic fluid throughout the 4 days; interestingly, exposure to ascitic fluid even after

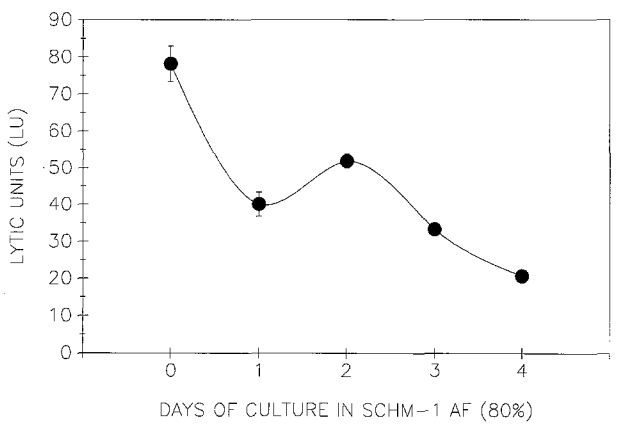

Fig. 1. Time course assay of LAK generation in SCHM-1 ascitic fluid (*AF*) (80% v/v). Peripheral blood mononuclear cells were grown with 100 U IL-2/ml in either R-10 for 4 days, or in R-10 for 1, 2 or 3 days and transferred to 80% SCHM-1 AF for the remainder of the culture period or were cultured in SCHM-1 AF for 4 days

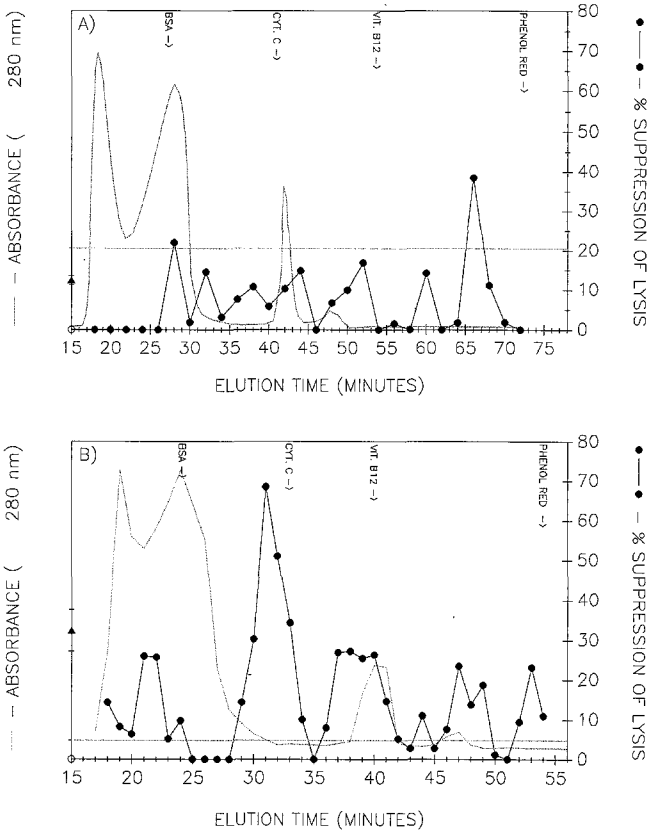

Fig. 2 A, B. Effect of HPLC fractionated SCHM-1 ascitic fluid (AF) on LAK generation. Fractions where control markers: bovine serum albumin (*BSA*) 63 kDa, cytochrome *c* (*CYT. C*) 12.5 kDa, vitamin B_{12} (*VIT. B12*) 1.355 kDa, and phenol red 0.375 kDa) eluted are as indicated. **A** – – –, Absorption spectrum (A_{280}) of 5 × concentrated SCHM-1 AF. ·–·, Suppression (%) of specific lysis of Daudi targets (compared to 20% TRIS buffer control) when PBM were incubated with HPLC fractions (20% v/v) for 4 days and tested at an effector-to-target ratio of 10:1. ○, Suppression (%) of specific lysis ±2 SEM obtained with PBM incubated in 20% TRIS buffer control. ▲, Suppression (%) of specific lysis obtained with PBM incubated in 20% whole SCHM-1 AF ± 1 SEM. **B** – – –, Absorption spectrum (A_{280}) of 5 × concentrated SCHM-1 AF. ·–·, Suppression (%) of specific lysis of Daudi targets (compared to 25% 0.58% glacial acetic acid in 0.1 M KCl buffer control) when PBM were incubated with HPLC fractions (25% v/v) for 4 days and tested at an effector-to-target ratio of 10:1. ○, Suppression (%) of specific lysis ±2 SEM obtained with PBM incubated in 25% 0.58% glacial acetic acid in 0.1 M KCl buffer control. ▲, Suppression (%) of specific lysis obtained with PBM incubated in 25% whole SCHM-1 AF ± 1 SEM

3 days of culture led to a significant reduction in LAK activity by day 4.

As a further test of the ability of ascitic fluid to arrest an established LAK-generating proliferative response, PBM were grown in the presence of 100 units IL-2/ml in R-10 for 4 or 8 days, R-10 for 4 days followed by ascitic fluid for 4 days or for 8 days (Table 7). Cells grown in R-10 for 8 days had the highest lytic activity (72.9 ± 2.2 LU) whereas cells grown in SCHM-1 or HUG-2 ascitic fluid for 8 days were the least active (15.2 ± 0.8 and 0.0 LU respectively). Those grown in SCHM-1 and HUG-2 ascitic fluid, after the initial 4-day culture in R-10, maintained the majority of the lytic activity generated by day 4 (75.9% and 87.6%), and had significantly greater lytic activity compared to PBM cultured in ascitic fluid for 8 days

Table 8. Effect of neutralizing anti-(transforming growth factor β) (TGFβ) antibody on suppressive activity of acid HPLC elution fractions

Sample	Specific lysis (%) ± 1 SEM
Buffer control	56.1 ± 2.3
Negative fraction (no. 28)	
+ medium	54.1 ± 2.1
+ rabbit IgG	55.1 ± 2.1
+ anti-TGFβ	57.7 ± 4.0
Positive fraction (no. 31)	
+ medium	43.8 ± 1.3*
+ rabbit IgG	47.9 ± 2.5*
+ anti-TGFβ	58.7 ± 3.8

* $P < 0.05$ for suppression compared to buffer control by Student's *t*-test

Table 9. Effect of neutralizing anti-TGFβ antibody on suppressive activity of whole ascitic fluid

Sample	Specific lysis (%) ± 1 SEM
Control medium	56.1 ± 2.3
HUG-2 ascitic fluid	
+ medium	47.1 ± 1.5*
+ rabbit IgG	45.0 ± 5.2*
+ anti-TGFβ	69.5 ± 5.7
SCHM-1 ascitic fluid	
+ medium	38.2 ± 7.4*
+ rabbit IgG	42.1 ± 0.8*
+ anti-TGFβ	58.0 ± 2.0

* $P < 0.05$ for suppression compared to control medium by Student's *t*-test

(30.0 ± 0.1 vs 15.2 ± 0.8 LU for SCHM-1 and 34.6 ± 0.1 vs 0.0 LU for HUG-2). However, expansion of lytic activity of activated LAK subsequently grown in ascitic fluid was impaired compared to the activity generated by culture in R-10 for 8 days (41.2% for SCHM-1 and 47.5% for HUG-2). Thus, ascitic fluid, in the presence of IL-2, allowed maintenance of the majority of LAK activity once generated but appeared to prevent further enhancement of this activity.

To characterize the factor(s) responsible for suppressive activity in ascitic fluid, SCHM-1 ascitic fluid was fractionated by HPLC (Fig. 2). Figure 2A shows that fractionation using TRIS buffer at pH 7.0 led to low recovery of suppressive activity from the HPLC with relatively small peaks of suppression eluting after 27–28 min (corresponding to a molecular mass of 60 kDa and after 65–66 min (corresponding to a molecular mass of 0.5 kDa). This suggested that activity might be sticking to the column (reminiscent of TGFβ). Therefore, elution was done using 0.1 M KCl 0.58% acetic acid. Figure 2B shows a major suppressive peak eluting after 29–35 min (corresponding to a molecular mass of approximately 25 kDa) as well as several smaller peaks of suppressive activity eluting after 36 min (corresponding to a molecular mass of <1500 Da). Neutralizing anti-TGFβ antibody was able to reverse the inhibitory activity of these fractions (Table 8). Similar studies done with two samples of unfractionated ascitic fluid showed that the suppressive activity of whole ascitic fluid was also reversed (Table 9). Thus in the samples tested, the major suppressor activity appears to be TGFβ.

Discussion

The data presented in this paper show that ascitic fluid from patients with ovarian carcinoma can inhibit the generation of LAK activity. The suppressive activity of ascitic fluid could arrest an ongoing LAK response within 24 h of exposure. This suggested that the effect might be at the step of activation of lytic activity (rather than proliferation), which for LAK requires at least 48 h of culture in the presence of IL-2 [10, 29]. However, ascitic fluid did not have a suppressive effect on pre-formed LAK activity and therefore did not deactivate LAK cells. Increasing the concentration of IL-2 up to 1000 units/ml in ascitic fluid only partially reversed its inhibitory activity. This IL-2 concentration appears to be in the upper range that can clinically be achieved without undue toxicity [37], even when given locally (i.e. intra-peritoneally).

Although only minimal suppressive activity was detected on elution of ascitic fluid at neutral pH from HPLC, high-potassium acid HPLC buffer eluted a dominant 25-kDa peak of suppression as well as several smaller peaks of low-molecular-mass (<1500 Da) suppressive activity.

Our findings are consistent with previous observations regarding the effect of TGFβ on the generation of LAK activity [11, 24]. TGFβ was found to suppress LAK induction and prevent IL-2-driven cytotoxicity. The effect on LAK was not immediate but developed over several days, and no effect on previously activated LAK was detected. In contrast to our studies, which revealed only minimal enhancement of LAK generation even at IL-2 concentrations of 1000 units/ml, it has been reported that the suppressive effect of TGFβ can be reversed by increasing IL-2 concentration [11]. Potential explanations for these effects produced by ascitic fluid are that very high levels of TGFβ may be present or that the TGFβ is associated with some high-molecular mass carriers that enhance its suppressive activity. Some low-molecular-mass inhibitors were also noted on HPLC and these may also be playing a role. The factors responsible for the suppressive activity in the low-molecular-mass fractions are presently under investigation.

TGFβ has been reported to be produced in a variety of tumors in vitro and by glioblastoma in vivo [1, 2, 41], although to our knowledge, not in association with ovarian carcinoma. A variety of reports have documented the presence of immunosuppressive humoral factors present in the serum or ascitic fluid of cancer patients. A number of these have described an acidic proteineic factor present in the ascites of ovarian carcinoma patients [3, 25, 34, 39]. Characterization of this substance revealed a protein of 50–52 kDa molecular mass composed of subunits of 25–26 kDa with the ability to inhibit spontaneous cytotoxicity of peripheral blood lymphocytes (PBL) against K562 cells. The nature of the 25-kDa subunits has not been determined. A molecule of 40–80 kDa, isolated from ovarian carcinoma ascites inhibited the blastogenic response of normal lymphocytes and blocked proliferation of both T

and B lymphoblastoid lines [14, 15]. A factor from malignant ascites causing dose-dependent suppression of DNA synthesis of phytohemagglutinin-stimulated donor PBL on further purification was isolated in a glycoprotein fraction of 440–1500 kDa molecular mass [9]. A natural killer cell (NK) inhibitory substance was found to be produced by peritoneal cells in patients with ovarian carcinoma. It was found to have a molecular mass of <2.0 kDa and was also able to inhibit antibody-dependent cellular cytotoxicity and NK activation by interferon α [20]. Finally, a low-molecular-mass protein (19 kDa) isolated from malignant effusions was found to suppress proliferation of the IL-2-dependent CTLL-2 cell line as well as the proliferative responses of human lymphocytes to concanavalin A or to allogeneic stimulation in mixed lymphocyte culture [4, 5, 13].

The effect of malignant ascitic fluid on the generation of LAK cells has been reported by a number of groups. Riley et al. [31] described two distinct inhibitors of LAK induction present in the ascites of a patient with ovarian carcinoma. IgG purified from the ascites reduced LAK activity as did normal human IgG as an aggregated form but not as a monomeric form. It is possible the suppressive activity was actually due to TGFβ adherent to IgG. Fractionation of IgG-free ascites revealed a second inhibitor of 65 kDa molecular mass. Interestingly, our data (Fig. 2A) suggests a similar inhibitor; this could also represent TGFβ that has not been freed from its carrier. Taylor et al. [40] demonstrated suppression of LAK generation when PBL were cultured in the presence of malignant effusion fluid (10% v/v). Suppression was detected in eight of ten effusions tested, including one of two samples of ascitic fluid from ovarian carcinoma. Suppressive activity occurred with as little as 2 h of pre-incubation of effectors with effusion fluid. In contrast, our studies revealed that the magnitude of the effect increased over the 4 days of incubation. On fractionation the suppressive activity studied by Taylor eluted at a molecular mass of >200 kDa; however, the elution buffer and columns used were not described.

An important issue concerns whether or not suppression by ascitic fluid occurs in situ. Steis et al. [37] reported that, in patients treated with intraperitoneal LAK plus IL-2, the ability of cells from the peritoneal cavity to lyse Daudi targets persisted for the duration of IL-2 administration. Stewart et al. [38] were able to generate peritoneal LAK cell activity in eight patients on an i.p. LAK/IL-2 protocol. Peritoneal LAK cell activity (against Daudi targets) was detected as early as day 3 or 4 in four patients, by day 11 in all eight patients and persisted as long as day 19. These cells lost lytic activity by 48 h in culture without IL-2. Hence, none had factors in their ascitic fluid that could completely inhibit LAK generation (consistent with our results). However, it is not clear that the LAK activity obtained in these patients was maximal. The peritoneal fluid LAK activity measured by Steis [37] was maintained but it is not clear whether there was an increase in activity. Djeu et al. [8] demonstrated that effusion-associated lymphocytes present in ascitic or pleural fluid from 15 cancer patients (ovarian or breast) could be activated by IL-2 in the presence of autologous effusion fluid, to lyse the NK-resistant but LAK-sensitive FMEX melanoma cell line. Similarly, Sone et al. [36] demonstrated that when mononuclear cells from pleural effusions of lung cancer patients were cultured with IL-2 in the presence of cell-free effusion fluid, LAK activity against Daudi targets was generated. Further, IL-2 injected daily intra-pleurally into malignant effusions resulted in in vivo induction of LAK activity. Taken together, such studies suggest that it is possible to generate LAK activity in the presence of ascitic fluid in vivo. What is not clear is whether the activity generated in vivo is maximal and whether it is sufficient (i.e. are the LAK cells generated capable of killing the tumor in the patient rapidly enough and are there a sufficient number?). In preliminary experiments, we have found PBM from patients with ovarian carcinoma, when cultured in 100 units IL-2/ml in the presence of autologous ascitic fluid, have reduced lytic activity compared to PBM growth in its absence. This is consistent with the results obtained using allogeneic PBM and suggests that the results obtained with allogenic PBM are relevant to the autologous situation.

In addition to suppressive factors, ascitic fluid may also contain factors that could enhance LAK generation [26, 27]. This is suggested by results obtained by adding ascitic fluid to LAK cells generated under sub-optimal conditions (Table 8, AIM-V medium +50% NAS-12 ascitic fluid) or under conditions where suppressive factors may have been diluted sufficiently to allow expression of factors enhancing LAK generation (Table 4, NAS-12 ascitic fluid at a 10% dilution in R-10).

The question still to be answered is why LAK cells appear to be universally effective against a broad range of fresh and cultured tumor targets and can be generated in vitro, and in situ with IL-2, yet overall response rates using LAK and IL-2 therapy in clinical trials remain disappointingly low [6, 32, 33]. Potential explanations include the much larger tumor bulk encountered in vivo (compared to single-cell suspensions tested in vitro) and target-cell-directed inactivation of LAK [42] preventing "multiple-hit" lysis in vivo. Tumoricidal activity and motility may be independently and perhaps inversely regulated [30], so in vivo LAK activity may reflect the activity of cells that are the least motile (but have the greatest tumoricidal activity). Conversely, those LAK cells capable of infiltrating tumors and contacting the greatest number of targets may be the least cytotoxic. Finally, the tumor micro-environment may also play a role in modulating LAK generation. It has been shown that although moderate alterations in oxygen, pH or glucose produce only slight impairment of lytic activity, LAK cell proliferation is markedly decreased under moderately low pH and low glucose conditions, which might be found in solid tumors characterized by discontinuities in vascular perfusion [21]. The effective concentration of TGFβ may be much higher in the local environment of the tumor compared to ascitic fluid.

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