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Immunotherapy of Ovarian Cancer

II. *In Vitro* Generation and Characterization of Lymphokine-Activated Killer T Cells from the Peripheral Blood of Recurrent Ovarian Cancer Patients[1]

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We examined the *in vitro* sensitivity of continuous ovarian cancer cells to lymphokine-activated killer T cells (T-LAK) alone or in combination with cytokines. Lymphocyte viability in T-LAK cultures generated from normal donors and ovarian cancer patients declined in the first 2 to 4 days; however, the remaining cells in these cultures maintained a constant rate of proliferation for long periods *in vitro*. These cells became 90–95% $CD3^+$ TCR^+ $-\alpha/\beta$ T-cells after 7–10 days in culture. The T-LAK cells from normal donors and cancer patients expressed an equal ability to induce lysis of a panel of human target cells (NK-sensitive K562, NK-insensitive RAJI, and two human ovarian tumor lines, SKOV-3 and OVCAR-3), demonstrating that they are nongenetically restricted killers. Preincubation of either the effector or target cells with tumor necrosis factor or interferon-γ or addition of these cytokines directly to cytolytic assays did not alter the degree of cell lysis *in vitro*. This is a method for generating large numbers of autologous, cytolytically active T-LAK cells from the blood of ovarian cancer patients that could be employed in adoptive intraperitoneal immunotherapy. © 1992 Academic Press, Inc.

INTRODUCTION

Ovarian cancer is the leading cause of death among gynecologic malignancies. Patients with ovarian cancer are logical candidates for intraperitoneal (ip) immunotherapy because this form of cancer remains confined to the peritoneal cavity until very late in the disease process and resistance to chemotherapy is frequently encountered. Thus, the tumor is readily accessed via ip treatment, which avoids one of the major problems in immunotherapy, which is delivering the agent(s) to the tumor site(s). It is therefore important to establish the sensitivity of human ovarian cancer cells to cytokines and host effector cells that can have antitumor activity *in situ*. This knowledge could be valuable in the design and implementation of clinical trials that employ these agents in the treatment of ovarian cancer.

Studies from multiple laboratories [1,2], including ours [3], have established that primary and continuous ovarian cancer cells are quite resistant to single- or multiagent exposure of tumor necrosis factor (TNF), lymphotoxin (LT), and interferons (both IFN-α and IFN-γ) *in vitro*. Its also been shown that both primary and continuous ovarian tumor cells are resistant to natural killer (NK) cells isolated from peripheral blood of normal donors and ovarian cancer patients [4]. In contrast, they can be lysed by lymphokine-activated killer cells (LAK) of NK cell origin after they have been cultured for 3–5 days with interleukin-2 (IL-2) [4,5]. However, there is evidence that this class of cells from the ascities of ovarian cancer patients may not be as effective as LAK cells derived from normal individuals [6]. In support of this, it has been reported that peritoneal ascites from ovarian cancer patients contain material(s) that suppresses IFN-γ-induced activation of NK-LAK cells *in vitro* [7].

The $CD3^+$ T cell is an important effector cell in the peritoneal cavity of woman with this disease. $CD3^+$ T cells predominate in the ascites fluid and the cellular infiltrate in solid nodules of tumor from these patients [8,9]. T cells from these tissue sources can be activated with IL-2 *in vitro* to become nongenetically restricted T-LAK

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cells [10]. It is the $CD3^+$ population of T cells that increases and expresses LAK cell activity *in situ* in the peritoneal ascities of woman with ovarian cancer who are receiving ip IL-2 immunotherapy [11,12]. While the cell lytic capacities of the T-LAK cells derived from ascites or infiltrating tumor nodules in these cultures varied, they are nongenetically restricted killers and are capable of inducing lysis of both primary and continuous ovarian cancer cells *in vitro.* [9,10] Finally, its been shown human NK-LAK and T-LAK cells, activated *in vitro* with IL-2, can increase survival when injected ip into nude mice bearing an ip human ovarian carcinoma [13].

Collectively, these studies indicate that ovarian cancer cells are quite resistant to cytokines but susceptible to LAK cells derived from both NK and T lymphocytes *in vitro.* While there are studies examining LAK cells of NK and T origin from other tissue sources, there are no studies to establish whether T-LAK cells can be generated from the peripheral blood of ovarian cancer patients and whether they are as effective as T-LAK cells from normal woman. The purpose of this study was twofold: to produce T-LAK cells from peripheral blood of these patients and to establish the *in vitro* cytolytic activity of these cells alone and in the presence of cytokines.

MATERIALS AND METHODS

1. Recombinant Cytokines

The following cytokines were used: recombinant human interferon-γ (Biogen Research Corp., Cambridge, MA) at 1.9 mg/ml, specific activity, 3.4×10^7 U/mg in 5% sucrose in phosphate-buffered saline (PBS) (0.15 *M* NaCl, 10 m*M* $NaHOP_4$, pH 7.2), and recombinant human tumor necrosis factor α (Genentech, South San Francisco, CA), specific activity, 5.6×10^7 U/mg. The cytokines were diluted in RPMI 1640 (GIBCO Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS) (GIBCO) (RPMI-10%).

2. Continuous Cell Lines

Four continuous human cell lines were employed as target cells in these studies: (A) RAJI, a NK-insensitive B lymphoblastoid cell line; (B) K-562 (K562), a NK-sensitive erythroleukemic cell line; and (C) two ovarian carcinoma cell lines, NIH:OV-CAR-3 (OVCAR-3) and SK-OV-3 (SKOV-3) (American Type Culture Collection, Rockville, MD). These cell lines were maintained in RPMI-10% in an atmosphere of 95% air and 5% CO_2. The OVCAR-3 and SKOV-3 cell lines were maintained as monolayer cultures in T-75-cm^2 tissue culture flasks and passed biweekly. The culture media for the OVCAR-3 was supplemented with 10 μg/ml bovine insulin (Sigma), which was necessary to support its growth. The K562 and RAJI cell lines were grown as suspension cultures in T-25-cm^2 tissue culture flasks and passed three times a week.

3. Collection of Peripheral Blood Mononuclear Cells (PBM)

Patients (21) ranged in age from 41 to 86; 20 had stage III and 1 had stage IV disease. Fourteen patients had received prior chemotherapy and seven had received no prior chemotherapy. The age of normal female donors ranges from 21 to 30. These individuals were seen either at the Memorial Cancer Institute or at the University of California, Irvine Medical Center.

Peripheral blood was collected from patients or normal donors by venipuncture. The heparinized blood (30–60 ml) was diluted with an equal volume of PBS and separated by density gradient centrifugation on 1.077 Histopaque (Sigma, St. Louis, MO), according to the method of Boyum [14]. The PBM cells were collected from the interface of the plasma and Histopaque and further washed to remove the remaining Histopaque by alternate centrifugation (500 *g* for 10 min) and resuspension in AIM-V (Gibco) supplemented with 2% FCS (AIM-V 2%). After the final centrifugation the cell pellet was resuspended in AIM-V 2% and the cells were counted using a Spotlite hemacytometer (American Scientific Products, McGaw Park, IL). These cells were 99 to 100% viable as determined by eosin Y staining.

4. Production of Lymphokine-Activated Killer T Cells

Human PBM cells were initially cultured in Aim-V 2% supplemented with 400 U/ml of recombinant human IL-2 (Cetus Corp., Emeryville, CA), and 0.4 μg/ml phytohemagglutinin-P (PHA-P) (Sigma). The T-LAK cells were cultured by a method similar to that described by Ingram *et al.* [15], at a cell density of 2×10^6 cells/ml in a T-75-cm^2 tissue culture flask. Adherent cells were present in these cultures for the first 48 hr; however, they were discarded upon subsequent subculture. After 48 to 72 hr at 37°C a cell sample in 0.1% eosin Y was counted in a Spotlite hemacytometer and resuspended at 0.5×10^6 viable cells/ml in fresh Aim-V 2% containing 400 U/ml of IL-2. These T-LAK cell cultures were recultured every 48 hr in fresh AIM-V 2% and 400 U/ml IL-2 at a density of 0.5×10^6 cells/ml.

5. Cytolytic ^{51}Cr Release Assay

Cytolytic assays employing the nonadherent target cells RAJI and K562 were performed in 96-well round-bottomed microcytotoxicity plates, whereas cytolytic assays for adherent ovarian cell lines were performed in 96-well flat-bottomed microcytotoxicity plates. These assays have been previously described [16]. Radiolabeling of the non-

adherent target cells was accomplished by the addition of 20 μl of ^{51}Cr (ICN, Irvine, CA) (specific activity, 183 mCi/mg; concentration, 5 mCi/ml) to 10^6 cells in 1 ml of serum-free RPMI for 2 hr at 37°C. The cells were then washed three times by alternate centrifugations (300 *g* for 5 min) and resuspensions in RPMI-10%. A constant number of labeled target cells (2×10^4 in 20 μl) was added to microtiter wells with various numbers of effector cells. Radiolabeling of adherent target cells was accomplished by the addition of 25 μl of ^{51}Cr to 1×10^6 cells in 10 ml of RPMI-10%. The radiolabeled cell suspension was then dispensed at a 100 μl/well in the flat-bottomed microtiter plate. After incubation for 24 hr at 37°C, the labeled cells were washed with RPMI-10% to remove free isotope. Effector cells were added to the target cells at various effector-to-target-cell (E:T) ratios. Both nonadherent and adherent target cell cytolytic assays were incubated at 37°C for 4 hr in an atmosphere of 5% CO_2. The release of ^{51}Cr was measured by the uptake of cell-free supernatants with Titerteks Supernatant Collection System (Skatron, Lier, Norway) and quantitated in an automated Clini-gamma counter (Pharmacia LKB Biotech Inc., Piscataway, NJ). The total ^{51}Cr releasable (90–95% of total counts) was determined by lysing the cells with 100 μl of 3% (w/v) sodium dodecyl sulfate solution. Spontaneous ^{51}Cr release was 1–2% per hour. Percentage of lysis was determined by the formula

$$\frac{\text{Experimental Release} - \text{Spontaneous Release}}{\text{Total Release} - \text{Spontaneous Release}} \times 100 = \%\ \text{Specific Lysis.}$$

6. Flow Cytometric Analysis

Cell suspensions were analyzed with single color analysis by FACScan (Becton–Dickinson, Mountain View, CA). The FACScan was calibrated with the Autocomp program and optimized for each sample tested. Forward scatter thresholds were set to eliminate debris and dead cells. The subthreshold particles were less than 5% of the total population of cells, which is in agreement with dye exclusion studies of these same cultures. Cells were optimized using forward vs side scatter to obtain a uniform distribution. Cells stained with isotypic control antibodies were used to determine background fluorescence. Surface phenotypes on lymphocytes were analyzed by staining the cells for surface antigens with the following monoclonal antibodies: (a) CD3 (Pan T), (b) CD4 (helper-inducer), (c) CD8 (cytotoxic-suppressor), (d) CD16 (natural killer) (e) TCR-α/β-1 (human T-cell antigen receptor α/β), (f) TCR-γ/δ-1 (human T-cell antigen receptor γ/δ). The monoclonal antibodies were fluorescein or phycoerythrin conjugated and were obtained from Becton–Dickinson Immunocytometry Systems. Cells were incubated with individual monoclonal antibodies for 30 min on ice and washed twice with PBS by alternate centrifugation (5 min at 300 *g*) and resuspension. Cells were fixed in 1.0% paraformaldehyde solution and stored at 4°C until further analysis. Data were expressed as percentage of positive cells for corresponding surface antigen.

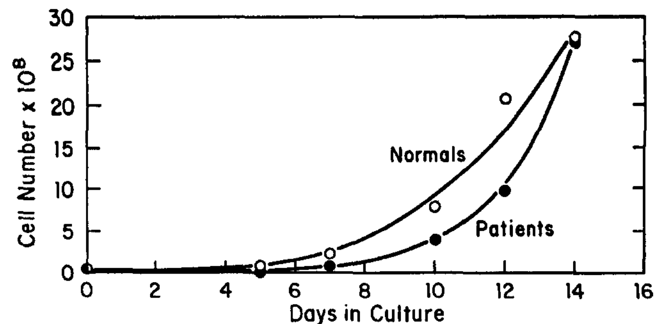

FIG. 1. The growth of peripheral blood mononuclear cells (PBM) from ovarian cancer patients and normal donors *in vitro*. PBMs were established in culture with PHA-P and IL-2 for 48 hr, and thereafter for IL-2 only, as described under Materials and Methods. The average cell number from individual cultures of three ovarian cancer patients and three normal donors was calculated on Days 5, 7, 10, 12, and 14.

RESULTS

1. Growth of PHA-Induced Peripheral Blood Mononuclear Cells in IL-2 from Ovarian Patients and Normal Donors in Vitro

Cultures of PBMs were established at a density of 2×10^6 cells/ml in T-75-cm^2 flasks in Aim-V 2%. The initial cultures contained 0.4 μg/ml of PHA and 400 U/ml of IL-2. Total viable cell numbers were determined every 48–72 hr when cultures were expanded. Over a 12-month period PBM cells from 12 normal women and 21 patients with ovarian cancer were collected and expanded in culture. The growth patterns of cells from 3 cancer patients and 3 normal individuals over a 14-day period were determined. Standard curves for these patients and normals were derived by summation and the data are shown in Fig. 1. The growth curves of the remaining 18 patients and 9 normal individuals were not significantly different from the data shown. There is a decline in viable cell numbers over the first 2–4 days in all these cultures. However, the cells begin to proliferate between 3 and 6 days and maintain a constant 18- to 20-hr division time. Most cultures were terminated after 2–4 weeks; however, it was possible to maintain many for up to 2 months. We detected no significant differences in growth patterns between cultures derived from normal and those from tumor patients. These T-LAK cells from either normal or tumor patients would not proliferate in the absence of IL-2.

2. Expression of Lymphocyte Surface Markers by PBM and T-LAK Cells Derived from Normal Individuals and Tumor Patients

The PBM and T-LAK cells from tumor patients and normal donors were subjected to phenotyping at various intervals during the culture period. Table 1 is a summary derived from phenotyping PBMs and 7- to 14-day T-LAK cells from three normal and three tumor patients. The results shown are representative of the data from all normal individuals and tumor patients examined. While not shown, sequential studies revealed that $CD3^+$ T cells predominate (90–95%) these cultures by 5–7 days and for the remainder of the culture period. These phenotypes remained relatively unchanged even in cultures maintained for long periods. However, the ratio of CD4 to CD8 cells varied from donor to donor. The numbers of cells expressing CD16 are a small percentage (5–10%) of the T-LAK cell population and remained low for the remainder of the culture period.

3. In Vitro Cytolytic Activity of 7- to 14-Day T-LAK Cells Derived from Normal Individuals and Ovarian Cancer Patients

T-LAK cells in these cultures were tested for their capacity to induce cytolysis of a panel of continuous cell lines *in vitro*. Four-hour ^{51}Cr release assays were conducted against two ovarian cell lines and two leukemic cell lines at various lymphocyte E:T cell ratios as described under Materials and Methods. The data presented in Table 2 are representative of the data obtained from extensive studies and show the results from three tumor patients and three normal donors. For clarity, only the mean of triplicate wells is shown in each table; however, variation was from 3 to 10% between triplicate wells. Individual T-LAK cultures from ovarian cancer patients and normal donors varied greatly in their ability to lyse these cells; however, when the average percentage of lysis of all the cultures was determined, we saw no significant difference between patient and normal T-LAK cultures (Table 3). The cytolytic activity of each culture was relatively stable over the entire 14-day culture period and then declined slowly with further culture.

TABLE 2
Percentage of Specific Lysis of Target Cells by T-LAK Cells in a 4-hr ^{51}Cr Release Assay *in Vitro*

Target cell	E:T ratio	Normal			Patient		
		1	2	3	1	2	3
OVCAR-3	25:1	68	85	73	49	81	23
	10:1	32	49	47	46	67	11
	5:1	8	18	27	30	51	11
SKOV-3	25:1	34	39	34	36	66	78
	10:1	10	12	11	26	48	37
	5:1	1	5	1	14	21	32
K562	25:1	63	68	59	78	71	88
	10:1	39	43	27	58	68	72
	5:1	25	26	14	36	38	60
RAJI	25:1	53	31	33	25	67	12
	10:1	29	20	18	16	45	17
	5:1	19	13	14	13	31	4

4. The Effects of TNF and IFN-γ on the Lysis of Continuous Ovarian Cancer Cell Lines by T-LAK Cells in Vitro

First we examined the ability of various doses of TNF and IFN-γ either singly or in combination to affect T-LAK cell cytolytic activity when added directly to the cell-killing assay. Concentrations of cytokines ranging from 10 to 1000 ng/ml were tested in this assay. When added directly to the killing assay, all the different concentrations and combinations of TNF and IFN-γ failed to increase the cytolytic activity of T-LAK cells on either the OVCAR-3 or the SKOV-3 cell lines *in vitro* (data not shown).

Next, studies were designed to test whether preexposure of either the effector T-LAK cell or the target cell or both to cytokines made the T-LAK cell a better killer or made the target cell more susceptible to lysis *in vitro*. A summary of the results obtained from two recurrent ovarian cancer patients and two normal donors is shown in Table 4. For simplification, only one E:T ratio (20:1), one concentration of cytokines (10 ng/ml), and one preincubation time (24 hr) are shown. While not shown, one other E:T ratio was tested (5:1); two other concentra-

TABLE 1
Expression of Phenotypic Markers on Fresh PBMs and 7- to 14-Day T-LAK Cells from Normal Donors and Tumor Patients

Cells tested	$CD4^+$	$CD3^+$	$CD8^+$	$CD16^+$	TCR-α/β	TCR-γ/Δ
			Normals			
PBMs	42 ± 9	81 ± 5	36 ± 19	16 ± 4	—	—
T-LAKs	30 ± 10	95 ± 4	61 ± 15	9 ± 7	92 ± 3	0
			Patients			
PBMs	42 ± 14	70 ± 17	36 ± 4	15 ± 15	—	—
T-LAKs	33 ± 13	96 ± 6	59 ± 10	8 ± 7	93 ± 2	0

TABLE 3
Percentage of Specific Lysis of Target Cells by T-LAK Cells in a 4-hr ^{51}Cr Release Assay[a] *in Vitro*

Target cell	Normal (n)	Patient (n)	P
K562	58 (12)	60 (18)	NS
RAJI	28 (12)	39 (9)	NS
OVCAR-3	70 (12)	57 (15)	NS
SKOV-3	36 (12)	50 (14)	NS

[a] The data presented are at an E:T ratio of 25:1 and are the averages of (n) donors.

tions of cytokines (100 and 1000 ng/ml) and two other preincubation times (48 and 72 hr) were also done. The most sensitive cell line, OVCAR-3, did show some increased lysis after treatment with TNF and IFN-γ in some of the normals tested, but these results were not seen consistently. In summary, all the studies showed that preexposure of the T-LAK cell and/or target cell to various concentrations and combinations of cytokines failed to significantly alter the cytolytic effectiveness of the T-LAK cells against the target cell.

DISCUSSION

In vitro studies from different laboratories indicate that both primary and continuous human ovarian cancer cell lines are relatively resistant to cytokines and peripheral NK cell derived from normal donors or tumor patients. However, these same cancer cells can be destroyed by LAK cells of NK- or T-cell origin. The NK-LAK cells were derived from patient peripheral blood and T-LAK cells were derived from patient ascites fluid and cells infiltrating solid tumor nodules [1–5,9,10]. The T cell may be an important host effector cell *in situ* in patients with ovarian cancer because $CD3^+$ T cells predominate in the ascities and in the cellular infiltrate in solid tumor metastasis in the peritoneal cavity of women with ovarian

TABLE 4
Percentage of Specific Lysis of Ovarian Cancer Cell Lines by T-LAK Cells when These Cells are Preincubated with TNF and/or IFN in a 4-hr ^{51}Cr Release Assay *in Vitro*

Target cell treatment[a]	LAK cell treatment[b] NT[c]	TNF	IFN	TNF + IFN	NT	TNF	IFN	TNF + IFN
	Patient 1				Patient 2			
OVCAR-3								
NT	44	32	37	37	44	33	49	34
TNF	29	41	30	35	41	56	86	31
+IFN	28	27	21	18	50	49	57	31
+TNF and IFN	43	23	33	28	53	39	39	29
SKOV-3								
NT	19	18	13	20	37	28	40	31
+TNF	19	17	15	25	50	34	56	26
+IFN	18	13	11	14	36	29	42	30
+TNF and IFN	24	17	16	17	44	37	47	36
	Normal 1				Normal 2			
OVCAR-3								
NT	16	19	19	11	16	8	19	17
+TNF	22	16	4	14	19	27	11	24
+IFN	28	18	9	24	21	20	23	18
+TNF and IFN	34	15	12	42	31	33	35	32
SKOV-3								
NT	3	2	1	3	5	3	2	4
+TNF	6	1	2	1	6	5	3	5
+IFN	7	4	2	3	4	4	1	5
+TNF and IFN	8	3	2	2	4	5	4	4

[a] The target cells were preincubated with 10 ng/ml of various cytokines for 24 hr.
[b] The T-LAK cells were preincubated with 10 ng/ml of various cytokines for 24 hr.
[c] Non treated

cancer [8,9]. In addition, the CD3$^+$ T-cell population increases and exhibits LAK activity in the ascites of women with ovarian cancer who are receiving ip IL-2 [11,12]. We were interested in establishing whether we could generate large numbers of T-LAK cells from the peripheral blood of women with this disease. This would be a more readily accessible source of effector cells than either ascites or tumor tissues.

The present studies show that T-LAK cells can be generated from the peripheral blood of women with ovarian cancer even after they have received chemotherapy. These cells proliferated readily for long periods *in vitro* in IL-2 after a short 48-hr stimulation with PHA-P and IL-2. The predominate cell in these cultures is a CD3$^+$/CD16$^-$ T cell. Even though there was variation in the CD4 to CD8 ratios from patient to patient, the T-LAK cells were capable of causing nongenetically restricted lysis of a panel of target cells, including continuous human ovarian carcinoma cell lines *in vitro*. We found no significant differences in growth characteristics, phenotype, or cell lytic ability between T-LAK cells derived from 21 patients with ovarian cancer and 12 normal women.

The capacity of TNF and IFN-γ to influence the cytolytic activity of 7- to 14-day T-LAK cells was also examined. When various concentrations and combinations of these cytokines were tested either directly in a cytolytic assay or were preincubated with the T-LAK or target cell they did not enhance the lytic effectiveness of these T-LAK cells. These results are in contrast to those reported by Waag *et al.* [17], who demonstrated that the presence of combinations of TNF and IL-2 in T-LAK cultures increased and sustained the *in vitro* cytolytic activity of CD3$^+$ tumor-infiltrating lymphocytes derived from solid ovarian cancer nodules cultured with IL-2. However, these cytokines exerted their effects only when they were present during the first few days of the culture period. Stotter *et al.* [18] found that TNF and IFN-γ increased the degree of *in vitro* cell lysis when target cells were preincubated for 24 to 72 hr with these cytokines. Why TNF and IFN-γ had little effect in our studies is not clear; however, it is possible that these cytokines do not act on the CD3$^+$ T-LAK cells after they have been stimulated and cultured in IL-2 for 7–14 days, but can affect these cells when present at earlier stages of these cultures.

In summary, our studies reveal that this method has the potential to generate large numbers of CD3$^+$ T-LAK cells from the peripheral blood of women with ovarian cancer. These cells share phenotype and cytolytic activity with T-cells previously described in the ascites and infiltrating solid tumor metastasis in the peritoneal cavity of women with this disease. The peripheral blood is much more accessible and yields many more cells than either the ascites or tumor tissues from these patients. This technique could be used to produce large quantities of autologous T cells to be employed in intraperitoneal immunotherapy trials.

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