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Generation of stimulated, lymphokine activated T killer (T-LAK) cells from the peripheral blood of normal donors and adult patients with recurrent glioblastoma *

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Peripheral blood mononuclear cells (PBM) from normal donors and patients with recurrent glioma were activated initially for 48–72 h with phytohemagglutinin-P (PHA) and recombinant human interleukin-2 (IL-2), and then proliferated in vitro for up to 5 months with IL-2. These cells are termed mitogen-stimulated lymphokine-activated T killer (T-LAK) cells. We measured patterns of T-LAK cell growth, in vitro cytolytic activity on a panel of continuous and primary tumor cells, and the phenotypes of the cells in these cultures. Lymphocyte viability declined dramatically over the first 3–5 days; and then the remaining cells in these cultures began to divide and maintained a constant 30–36 h doubling time for long periods in vitro. Phenotyping revealed that cells in the initial few days of culture were heterogeneous, but became almost totally CD3 T cells after 7–10 days in culture. The T-LAK cells from individual normal donors and cancer patients demonstrated a non-genetically restricted cytolytic ability against a panel of both continuous cell lines and primary autologous and allogeneic glioblastoma cells in vitro. This technique provides a method of generating large numbers of autologous cytolytic T cells with non-restricted anti-tumor activity that can be derived from peripheral blood mononuclear cells.

Key words: Mitogen-stimulated lymphokine activated T killer cell; Recombinant human interleukin-2; Glioblastoma; Phenotyping

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Abbreviations: PBM, peripheral blood mononuclear cells; IL-2, recombinant human interleukin-2; T-LAK mitogen-stimulated lymphokine-activated T killer cells; LAK, lymphokine-activated cells; FACS, fluorescence-activated cell sorter; PHA, phytohemagglutinin.

Introduction

Peripheral blood lymphocytes from normal human donors can be induced by co-culture with recombinant interleukin-2 (IL-2) (Grimm et al., 1982) or the lectins concanavalin A (ConA) or phytohemagglutinin (PHA) for 3–5 days to cause cytolysis of transformed cells in vitro (Mazumder

et al., 1982, 1983). The effector cells in these cultures have been termed lymphokine-activated killer (LAK) and PHA-activated killer (PAK) cells, respectively, and LAK cells are currently being tested in experimental immunotherapy of patients with different types of malignancies (Rosenberg et al., 1986).

Jacobs (1986) demonstrated that LAK cells obtained from human patients with recurrent glioma induce variable degrees of cytolysis of NK resistant cell lines and autologous and allogeneic glioma cells in vitro. Moreover, patients with recurrent glioma have been treated by intra-tumor implants of autologous LAK cells (Jacobs et al., 1986a,b,c; Merchant et al., 1986; Yoshida et al., 1988; Barba et al., 1989). Usually human LAK cells are generated by culture for 3–5 days with IL-2 (Grimm et al., 1982; Rosenberg et al., 1985). However, there are several reports in which patients with recurrent glioma were treated with autologous lymphoid cells pre-stimulated with PHA and cultured with IL-2 for 10-14 days (Ingram et al., 1987a,b; Kruse et al., 1989). The cells in these cultures were proliferating; however, the phenotype or capacity of these effectors to cause cytolysis of cells in vitro was not established. This report examines the growth characteristics, cell cytolytic capacity and phenotypic characteristics of the cells in these cultures. They are compared to unstimulated PBM and 3–5 day cultures of IL-2 stimulated LAK cells.

Materials and methods

Patient selection

Peripheral blood was collected from normal individuals between the ages of 26–53. The following were the characteristics of glioma patients: (a) age over 21; (b) histologic evidence of grade IV glioma; (c) prior treatment with chemotherapy and radiation therapy; (d) Karnofsky score of greater than 50; (e) adequate hematologic status; and (f) recurrent tumor of sufficient size, as determined by CT scan, to warrant a second surgery.

Mononuclear cells

Human peripheral blood was collected from patients or normal donors by venipuncture. Defibrinated blood (50–200 ml) was separated directly on 1.077 Histopaque (Sigma, St. Louis, MO), using density gradient centrifugation. Some subjects were treated by leukophoresis, using the Fenwal CS-3000 blood cell separator (Fenwal, Baxter Healthcare, Deerfield, IL).

Approximately 10^9 peripheral blood mononuclear (PBM) cells are collected from the phoresis of 1–1.5 liters of blood. Further elimination of red blood cell and platelet contamination was accomplished by separation on 1.077 Histopaque. The PBM cells are further washed to remove the remaining Histopaque by alternate centrifugation ($800 \times g$ for 10 min) and re-suspension in phosphate-buffered saline, or PBS (0.15 M NaCl, 10 mM $NaHPO_4$, pH 7.2). After removal of Histopaque, the cell pellet is resuspended in PBS and centrifuged at $150 \times g$ for 8 min. The supernatant is discarded and the cell pellet is subjected to this procedure twice more to remove as many of the platelets as possible.

Production of human lymphoid cell populations

Human PBM cells were initially cultured in Aim-V (Gibco Laboratories, Grand Island, NY) supplemented with 2% heat inactivated (1 h, 56°C) fetal calf serum (FCS) (Gibco). The LAK cell cultures received 200 U IL-2 (Hoffman-La Roche, Nutley, NJ), and T-LAK cell cultures received 0.4 μg/ml PHA-P (Burroughs-Wellcome Co., Research Triangle Park, NC) and IL-2. The T-LAK cells were cultured by a method similar to that described by Ingram (1987b), at a cell density of 2×10^6 cells/ml in either a T 150 cm^2 tissue culture flask or a 3 liter PL-732 Fenwal tissue culture bag. After 48–72 h in a 37°C humidified CO_2 incubator, a cell sample in 0.1% Eosin Y was counted in a Neubauer chamber by microscopic observation and resuspended, at 0.5×10^6 viable cells/ml, in fresh Aim-V containing 200 U/ml of IL-2. We refer to long-term cultures as cells that were maintained for longer than 10–12 days; short-term cultures are cells that were maintained for 3–4 days.

Target cells

Five continuous human cell lines were employed as target cells in these studies: (a) Raji natural killer (NK) insensitive B lymphoblastoid

cell line; (b) Molt-4 T lymphoblastoid cell and erythroleukemic K-562 NK-sensitive cell lines; and (c) two continuous glioblastoma cell lines, U-373 MG and U-138 MG. These cell lines were maintained in RPMI 1640 (Gibco), supplemented with 10% heat-inactivated FCS (RPMI 10%). The U-373 MG and U-138 MG cells were obtained from American type culture collection (Rockville, MD) and maintained as monolayer cultures in T 75 cm^2 tissue culture flasks in an atmosphere of 95% air and 5% CO_2, and passed biweekly. The suspension cultures of K-562, Molt-4 and Raji were grown in T 25 cm^2 tissue culture flask in RPMI 10% in an atmosphere of 95% air and 5% CO_2, and passed three times a week.

Culture of primary human brain tumor cells

Samples of human brain tumor tissue were obtained during surgery. The tumor sample was placed in a sterile petri dish and washed twice with Hanks' minimal essential medium (MEM). The tumor was then minced into small (1 mm) pieces and suspended in 2 ml of a 1/10 dilution of collagenase (cat. no. C-5894, Sigma, St. Louis, MO) in PBS. After 2 h at 37°C, the tissue fragments are vigorously pipetted. The cells and tissue fragments are suspended in MEM and pelleted by centrifugation at $300 \times g$ for 5 min. The supernatant is removed and the cell pellet resuspended in MEM supplemented with 20% FCS, 1% MEM non-essential amino acids, 1% MEM sodium pyruvate and 1% L-glutamine. The resulting cell suspension is added to a T 25 cm^2 tissue culture flask and incubated at 37°C in a CO_2 incubator. Monolayers were formed after 7–10 days and these cells were passaged by trypsinization and re-suspension in RPMI 10%. Once established, these cells divide rapidly and are passaged 2–3 times/week. These cells were of glial origin as judged by glial fibrillary acidic protein (GFAP) staining techniques.

Cytolytic ^{51}Cr release assay

Cytolytic assays employing the non-adherent target cells Raji, K-562 and Molt-4 were performed in 96 well round-bottomed microcytotoxicity plates, whereas cytolytic assays for adherent glial cell lines were performed in 96 well flat-bottomed microcytotoxicity plates. Radiolabeling of the non-adherent target cells was accomplished by the addition of 20 μl of ^{51}Cr with a specific activity of 183 mCi/mg (ICN, Irvine, CA), and a concentration of 5 mCi/ml to 10^6 cells in 1 ml of serum free media for 1–2 h at 37°C. After 1–2 h at 37°C, the cells were washed three times ($300 \times g$ for 5 min.) with cold 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 1.5×10^6 cells in 10 ml of RPMI 10%. The radiolabeled cell suspension was then dispensed at a 100 μl/well in the flat-bottomed microcytotoxicity plate. After incubation for 24 h at 37°C, the labeled cells were washed with cold RPMI 10% to remove free isotope. Effector cells were added to the target cells at various effector-to-target-cell ratios. Both non-adherent and adherent target cell cultures were incubated at 37°C for 4 h. The release of ^{51}Cr was measured by the uptake of cell-free supernatants with Titerteks supernatant collection system (Skatron, Norway) and quantitated in an automated Biogamma counter (Beckman, Fullerton, CA). 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%/h. Percent lysis was determined by the following formula:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

$$= \%\text{specific lysis}$$

Phenotypic analysis

Surface phenotypes on cultured and fresh lymphoid cells were analyzed by using monoclonal antibodies to CD3 (Pan T), CD4 (helper-inducer), CD8 (suppressor-cytotoxic), Leu-7 (T cell and NK cell subset), CD16 (Fc IgG receptor on NK cells and neutrophils), CD38 (thymocytes, NK cells, activated T cells, B cell subset), anti-HLA-DR (B cells, monocytes, macrophages, activated T cells) and CD25 (IL-2 receptors.) The monoclonal antibodies were either fluorescein or phycoerythrin conjugated and were obtained from Becton Dick-

inson (Mountain View, CA). Cells were incubated with desired monoclonal antibodies (according to the manufacturer's recommendation) for 30 min on ice and washed twice with PBS by centrifugation for 5 min at 1600 rpm. Cells were fixed in 0.5% paraformaldehyde solution and stored at 4°C until further analysis.

Flow cytometric analysis

Cells were analyzed by using FACScan (Becton Dickinson, Mountain View, CA). Cells were gated on forward vs. side scatter to obtain a uniform distribution and the green (PL1) or red (PL2) fluorescence was measured by analyzing the cells stained with FITC- or PB-labeled antibodies, respectively. Wherever appropriate, the dual fluorescence was analyzed. 10,000 cells stained with isotypic control antibodies were used to set markers for background fluorescence. Data were expressed as percent positive cells for corresponding surface antigen.

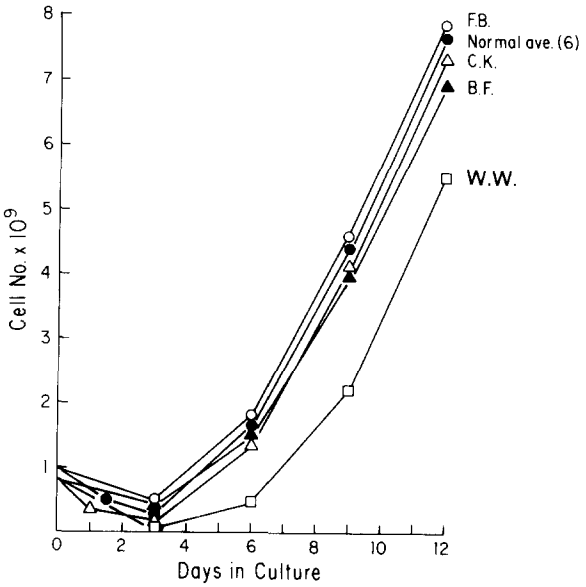

Fig. 1. The growth of human PBM after 48–72 h initial exposure to PHA and subculture in IL-2.

Results

Growth of PHA-induced PBM from normal individuals and recurrent glioma patients in IL-2 in vitro

Initially, cultures of PBM cells were established in small-scale cultures in T 75 cm^2 flasks and in large-scale cultures in Fenwal flasks in various media, including: (a) Aim-V, (b) X-Vivo, (c) HB 104, and (d) RPMI 10%. The initial cultures contained 0.4 μg/ml of PHA and 200 U/ml of IL-2. Total viable cell numbers were determined every 48–72 h when cultures were expanded. The data are not shown, but these studies revealed that cells proliferated faster in Aim-V than in any of the other media. Over a 12 month period PBM cells from 12 normal individuals and 15 patients with recurrent glioma were collected and expanded in small- and large-scale (up to 40×10^9 cells) culture. The growth patterns of the cells from four individual patients and a standard curve obtained by summation of the data from six normal individuals cultured over a 12-day period are shown in Fig. 1. The growth curves of the remaining 11 patients and six normal individuals were not significantly different from the data shown in that figure. There is a dramatic decline in viable cell numbers over the first 1–6 days in all these cultures. However, the cells begin to proliferate between 3–6 days and maintain a constant 30–36 h division time. Most cultures were terminated after 2–4 weeks; however, three normal and four patient cultures were maintained for 5 months. We detected no significant differences in growth patterns between cultures derived from normal and tumor patients. In addition, cells would not proliferate in the absence of IL-2.

In vitro cytolytic activity of PBM, LAK and T-LAK cells derived from normal individuals and patients with recurrent glioma tested on a panel of continuous cell lines

At various intervals, cells in these cultures were tested for their capacity to induce cytolysis of a panel of continuous cell lines in vitro. 4 h ^{51}Cr release assays were conducted as described in the materials and methods section. These data were very extensive. Cultures derived from 12 normal individuals and 15 patients were tested initially, at 3–5 days, and then weekly in triplicate at various lymphocyte effector : target (E : T) cell ratios. The data presented in Table I and Table II are representative of the data obtained from these studies and show the results from three tumor patients

and three normal donors all tested at the same time. To accomplish simultaneous testing of each type of effector cells, T-LAK cells were established; then PBM cells were collected and placed in culture with and without IL-2 three days prior to cytotoxic testing. All effectors were then harvested on the same day and tested simultaneously on each of the target cells. For clarity, only the mean of triplicate wells is shown in each table; however, variation was from 3% to 10% between triplicate wells. The data in Table III show the results of a single patient's cells tested over a 38 day culture period. These cells were not tested simultaneously, but data shown are from individual assays.

Unstimulated cells from normal and patient donors were poor or weakly lytic against the NK-sensitive K-562 and Molt-4 cells. In contrast, almost all effector cells were incapable of lysing NK-resistant Raji or continuous glioblastoma cells U373 and U138. Thus, these two glioblastoma cell lines appear to be resistant to NK cell-induced cytolysis. However, upon 3 days of co-culture with IL-2, LAK cells from most patients and normal individuals in these cultures gained the capacity to destroy all continuous target cells we employed. In a similar fashion, the T-LAK cells in these cultures also expressed the ability to kill all the continuous target cell lines. The cytolytic activity of LAK and T-LAK cells from patients and normal individuals was within the same range. However, the cells from both individual normals and patients varied widely in their actual cytolytic ability. For example, all effector cells from donor MF have weak cytolytic activity against NK-sensitive and NK-resistant targets. This was observed in many different experiments over a 5-month period with MF cells. While the data are not shown, a second cell culture was initiated from MF, 1 month after the first culture, and both LAK and T-LAK cells showed strong lytic activity against all targets for an entire 8 week culture

TABLE I

SPECIFIC IN VITRO ^{51}CR RELEASE FROM A PANEL OF CONTINUOUS CELL LINES INDUCED BY TREATMENT FOR 4 h WITH PBM, LAK AND T-LAK CELLS DERIVED FROM NORMAL INDIVIDUALS

Target cell employed	E:T [a]	^{51}Cr release induced by effector cells from								
		M.F.			T.J.			E.I.		
		PBM [b]	LAK [c]	T-LAK [d]	PBM	LAK	T-LAK	PBM	LAK	T-LAK
K-562	10:1	0.7	16.8	0.9	12.3	45.8	52.8	21.3	58.7	60.9
	5:1	0.2	6.9	1.4	9.7	26.7	37.2	12.4	37.3	40.2
	1:1	0.0	0.8	0.0	5.8	13.9	25.3	7.4	37.2	25.5
Molt-4	10:1	8.7	18.9	2.1	5.6	43.9	52.6	18.4	71.4	66.5
	5:1	3.6	6.4	0.6	2.5	31.5	39.6	13.9	56.8	41.9
	1:1	1.1	5.6	0.0	1.4	17.3	26.6	8.3	47.8	30.9
Raji	10:1	0.9	1.5	0.0	0.5	23.4	15.0	1.4	24.3	22.9
	5:1	0.0	0.0	0.0	0.4	16.7	8.4	0.8	12.2	9.9
	1:1	0.0	0.0	0.0	0.4	13.3	4.6	0.2	9.7	7.2
U-373	10:1	2.4	5.3	0.0	0.9	20.9	20.7	2.0	51.7	22.6
	5:1	1.9	2.7	0.0	0.0	8.8	12.3	0.6	31.5	16.3
	1:1	0.6	1.6	0.0	0.0	1.4	6.5	0.7	17.2	8.9
U-138	10:1	0.3	7.9	0.0	0.2	20.2	8.2	1.9	58.4	21.7
	5:1	0.0	6.1	0.0	0.0	7.7	0.0	1.2	32.1	18.8
	1:1	0.0	4.9	0.0	0.0	0.3	0.0	0.9	22.4	7.1

[a] Effector to target cell ratio.
[b] Unstimulated peripheral blood mononuclear (PBM) cells.
[c] LAK cells after 2–4 day co-culture with IL-2.
[d] T-LAK cells after 14 day co-culture with IL-2.

TABLE II

SPECIFIC IN VITRO ^{51}Cr RELEASE FROM A PANEL OF CONTINUOUS CELL LINES INDUCED BY TREATMENT FOR 4 h WITH PBM, LAK AND T-LAK CELLS FROM PATIENTS WITH RECURRENT GLIOMA

Target cell employed	E:T [a]	^{51}Cr release induced by effector cells from								
		F.B.			W.W.			B.F.		
		PBM [b]	LAK [c]	T-LAK [d]	PBM	LAK	T-LAK	PBM	LAK	T-LAK
K-562	10:1	18.0	75.3	65.1	21.9	86.5	70.1	9.5	57.5	81.6
	5:1	7.4	64.7	46.7	10.1	77.3	60.0	5.0	39.9	57.4
	1:1	1.3	41.9	21.7	5.4	55.8	42.3	2.1	22.4	31.3
Molt-4	10:1	25.8	78.2	61.2	38.5	72.7	74.3	14.2	63.3	73.0
	5:1	16.4	68.2	38.9	27.5	62.6	55.8	9.2	45.9	39.1
	1:1	8.3	54.4	22.6	13.2	44.3	35.0	4.5	28.3	24.2
Raji	10:1	4.9	63.4	50.6	31.3	100.0	73.6	2.7	48.9	82.6
	5:1	2.1	44.9	35.5	19.3	87.0	68.6	1.2	32.6	55.1
	1:1	1.4	24.9	18.8	5.9	58.4	42.8	0.7	18.9	33.1
U-373	10:1	0.0	84.5	52.9	21.2	27.4	46.7	9.4	33.3	22.0
	5:1	2.4	56.9	23.4	11.8	16.8	29.2	5.1	23.3	12.4
	1:1	1.6	27.1	13.4	2.2	8.5	9.9	3.7	10.1	7.7
U-138	10:1	7.9	85.6	51.3	23.0	38.0	37.9	0.0	43.0	23.4
	5:1	4.8	63.9	31.3	16.2	25.4	30.9	0.0	16.2	12.3
	1:1	1.0	42.1	10.3	3.5	10.1	15.1	0.0	0.0	3.7

[a] Same as in Table I.
[b] Same as in Table I.
[c] Same as in Table I.
[d] Same as in Table I.

period. The cytolytic activity of T-LAK cells from single donors was relatively stable in culture. The data in Table III show that cytolytic activity of cells from a single donor was expressed rather uniformly by these cells over the full culture period. In all experiments we found that the T-LAK cells did not change their functional capacity even in cultures maintained for as long as 5 months. Thus, cells which were poor killers remained poor killers, while cells which were highly effective killers remained good killers in vitro.

In vitro cytolytic activity of T-LAK cells derived from patients with recurrent glioma on autologous and allogeneic primary glioblastomas

Primary glioblastoma cultures were established from samples of patient tumors as described in the materials and methods section. IL-2-stimulated T-LAK cells from glioma patients were tested for their cytolytic activity and frozen at −70°C. After primary tumor cell lines had been established, they were also frozen at −70°C after 3–4 weeks of culture. Effector and target cells were thawed, cultured for 2–4 passages, and then simultaneously tested in identical fashion, as described in the previous section for continuous glioblastoma lines. The results of these experiments are shown in Table IV.

T-LAK cells from each patient were capable of inducing cytolysis of both autologous and allogeneic primary tumor cell lines. Thus, the T-LAK cells are not genetically restricted killers. However, there were differences in the lytic capacity of the cells from each donor T-LAK cells from all except one patient appeared to be effective at inducing cytolysis of most targets. In contrast, effector cells from R.L. appeared to be the least cytolytically active. Glioblastoma cells derived from C.K. were much more difficult to lyse by these effector cells from all donors than all the other glioblastomas.

TABLE III

SPECIFIC IN VITRO ^{51}Cr RELEASE FROM A PANEL OF CONTINUOUS CELL LINES INDUCED BY TREATMENT FOR 4 h WITH PBM, LAK AND T-LAK CELLS DERIVED FROM A SINGLE GLIOBLASTOMA PATIENT

Target cell employed	E:T [a]	PBM [b] 3 day	LAK [c] 3 day	T-LAK [d] 7 day	T-LAK [d] 11 day	T-LAK [d] 23 day	T-LAK [d] 38 day
K-562	10:1	21.9	86.5	90.9	70.1	80.2	90.5
	5:1	10.1	77.3	70.9	60.0	73.8	73.9
	1:1	5.4	55.8	31.5	42.3	61.3	53.6
Molt-4	10:1	38.5	72.7	79.5	74.3	75.1	84.8
	5:1	27.5	62.6	66.1	55.8	66.8	76.0
	1:1	13.2	44.3	29.4	35.0	58.9	64.7
Raji	10:1	31.3	100.0	56.1	73.6	48.2	45.1
	5:1	19.3	87.0	36.4	68.6	38.0	35.5
	1:1	5.9	58.4	14.2	42.8	34.9	23.3
U-373	10:1	21.2	27.4	75.6	46.7	54.9	35.7
	5:1	11.8	16.8	54.4	29.2	34.7	33.0
	1:1	2.2	8.5	23.2	9.9	14.1	18.4
U-138	10:1	23.0	38.0	60.0	37.9	32.1	ND [e]
	5:1	16.2	25.4	30.9	30.9	12.4	ND
	1:1	3.5	10.1	13.8	15.1	0.0	ND

[a] Same as Table I.
[b] Same as Table I.
[c] Same as Table I.
[d] Same as Table I.
[e] ND = not done.

Thus, glioblastomas can vary somewhat in their sensitivity to both autologous and allogeneic T-LAK cells.

Expression of lymphocyte surface markers by PBM, LAK and T-LAK Cells derived from normal individuals and patients with recurrent glioma

PBM, LAK and T-LAK cells from all normals and tumor patients were subjected to phenotyping at various intervals during the culture period. These studies were extensive. Shown in Table V are the data derived from typing the cells from three normal individuals and three patients. The results shown are representative of the data from all normal individuals and tumor patients examined. There are a number of changes evident in these cultures. IL-2 receptor-bearing cells were low in PBM but high in LAK cell population; then they dropped in the T-LAK cultures. The NK marker CD16 was reduced or absent in most 12–14 day cultures. However, the pan T cell marker CD3 was expressed on 90–97% of the cells in all 12 day cultures. Interestingly, CD8 and CD4 expressing cells alternated when CD8 T cells were high, CD4 T cells were low, and vice versa. Also, CD38 and anti-HLA-DR T cells were increased in T-LAK cells and remained high even in cells maintained for 5 months. Also, CD38 and HLA-DR cells were increased in LAK cells. The CD16 cells were significantly lower in the patients' LAK and T-LAK cells than in normal controls.

Discussion

Human PBM cells from normal donors and patients with recurrent glioma can be propagated for extended periods after pre-stimulation with PHA and co-culture with IL-2. About 50–80% of the cells in these cultures die during the first 2–4 days in vitro. This is the period in which LAK and PAK cells are usually harvested (Grimm et al.,

TABLE IV

SPECIFIC IN VITRO ^{51}Cr RELEASE FROM ALLOGENEIC AND AUTOLOGOUS PRIMARY GLIOBLASTOMA CELLS INDUCED BY T-LAK CELLS FROM GLIOBLASTOMA TUMOR PATIENTS

Target cell	E:T [b]	Patient T-LAK cells [a]				
		W.W.	R.A.	R.L.	C.K.	B.F.
K-562	25:1	84.2	90.3	69.1	80.2	86.5
	10:1	70.1	88.3	49.2	65.9	81.6
	5:1	60.0	82.1	33.1	48.0	57.4
W.W. glio-blastoma [c]	25:1	77.5 [d]	72.4	36.0	62.5	53.7
	10:1	53.1	67.5	17.3	43.8	31.8
	5:1	31.1	48.9	12.1	21.1	15.6
R.A. glio-blastoma [c]	25:1	47.9	38.9 [d]	47.4	77.0	43.3
	10:1	43.1	37.0	18.7	44.7	25.8
	5:1	31.1	26.5	14.9	26.2	19.6
R.L. glio-blastoma [c]	25:1	95.7	100.0	34.1 [d]	70.8	63.9
	10:1	67.3	87.3	20.3	55.4	38.6
	5:1	47.4	60.9	9.6	39.7	28.9
C.K. glio-blastoma [c]	25:1	24.8	34.7	10.8	37.8 [d]	32.1
	10:1	21.0	18.9	6.8	20.4	14.7
	5:1	9.1	10.6	5.7	9.3	12.7
B.F. glio-blastoma [c]	25:1	54.8	44.9	27.6	64.5	54.5 [d]
	10:1	49.0	41.2	15.0	40.3	25.6
	5:1	35.8	25.8	11.9	29.3	13.3

[a] 14 day T-LAK cells.
[b] Effector to target cell ratio.
[c] Primary human brain tumor cell line.
[d] Patient's own T-LAK cells tested on their autologous brain tumor cell line.

1982; Mazumder et al., 1982, 1983; Rosenberg et al., 1985; Jacobs et al., 1986a; Merchant et al., 1988). However, between 4–6 days, the cells in T-LAK cultures begin to proliferate in the presence of IL-2 and continue to divide at a constant rate, doubling every 24–30 h. While we terminated most cultures at 12–20 days, seven were continued for 5 months. All these cultures were vigorous up to 4–6 weeks; then two slowed and died out. However, the remaining six continued vigorous growth and expressed no change in functional activity for the entire culture period. Thus, lymphoid cells derived from PHA cells pre-stimulated PBM can be propagated in vitro for relatively long periods in the presence of IL-2. Ingram (1987b) employed similar induction and culture techniques but did not maintain cells beyond 14 days in vitro. Mazumder (1982, 1983) employed PHA and ConA to stimulate human PBL but collected the cells at 2–3 days and did not employ IL-2 to continue cell division. The present culture technique can provide large numbers of effector cells at the end of the culture period, or they can be harvested at intervals during the culture. While all the data are not shown, we found that these cells can be frozen at $-70°C$ and will express full cytolytic activity when thawed and retested in vitro.

Both LAK and T-LAK cells from normal individuals and recurrent glioma patients express varying degrees of cytolytic activity when simultaneously tested on a panel of continuous and primary cells in vitro. Unstimulated PBM cells from both glioma patients and normal individuals were lytic against the NK-sensitive K-562 and Molt-4, but not effective on NK-resistant Raji cells, and continuous and primary glioma cells. However, after 2–5 days, LAK cells in these cultures acquire the ability to lyse the NK resistant Raji cells and glioma targets, as has been previ-

TABLE V

EXPRESSION OF PHENOTYPIC MARKERS ON FRESH PBM, LAK CELLS AND T-LAK CELLS FROM NORMAL DONORS AND TUMOR PATIENTS [a]

		Monoclonal antibody employed							
		CD8	CD4	CD3	Leu-7	CD16	CD38	HLA-DR	CD25
Normal									
M.F. [e]	PBM [b]	ND [g]	ND	ND	ND	ND	ND	ND	ND
	LAK [c]	57.3	30.5	92.3	2.4	20.1	52.4	81.8	85.1
	T-LAK [d]	3.3	94.7	97.3	1.3	29.3	0.4	90.6	23.2
E.I. [e]	PBM	ND	ND	ND	ND	ND	ND	ND	ND
	LAK	75.8	6.1	95.2	70.1	60.6	54.2	95.0	5.0
	T-LAK	68.8	18.0	84.6	41.7	35.3	18.0	84.8	0.0
T.J. [e]	PBM	49.2	48.2	83.6	0.2	49.2	21.2	29.9	18.6
	LAK	42.3	12.7	66.5	0.0	52.6	46.3	33.1	54.1
	T-LAK	54.9	24.8	90.3	13.6	8.5	47.3	51.8	7.3
Patient									
F.B. [f]	PBM	14.0	55.0	89.0	20.0	25.0	25.0	19.0	13.0
	LAK	17.0	38.0	80.0	12.0	9.0	56.0	60.0	79.0
	T-LAK	46.0	10.0	97.0	40.0	3.0	34.0	91.0	23.0
W.W. [f]	PBM	13.9	63.3	64.9	9.3	41.5	13.4	17.8	0.5
	LAK	46.6	57.6	73.1	0.0	25.7	65.4	54.8	60.1
	T-LAK	66.1	30.0	96.8	28.9	75.4	25.6	67.7	11.2
B.F. [f]	PBM	31.0	51.0	87.0	23.0	2.0	12.0	38.0	9.0
	LAK	30.0	36.0	62.0	10.0	0.0	41.0	59.0	77.0
	T-LAK	29.0	57.0	90.0	12.0	0.0	44.0	89.0	41.0
Normal	range	28.0	45.0	75.0	20.0	15.0	–	11.0	–

[a] Cells were exposed to fluorescent tagged monoclonal reagents and then subjected to analysis by FACS as described in the materials and methods section.
[b] Same as in Table I.
[c] Same as in Table I.
[d] Same as in Table I.
[e] Normal donors.
[f] Glioblastoma patients.
[g] ND = not done.

ously reported by Jacobs (1986a,b). In a similar fashion, T-LAK cells from both normal individuals and glioma patients gained the ability to lyse NK-sensitive and NK-resistant targets and continuous and primary glioma cells in vitro. Cytolytic activity against NK-sensitive and resistant continuous cell lines has also been shown by Kruse, (1989), employing human PBM cultured and tested under similar in vitro conditions. However, our studies indicate that stimulation with PHA and co-culture with IL-2 did not raise T-LAK cells from all donors to the same levels of cell lytic effectiveness. It was surprising to see the wide variation in cell lytic activity of both LAK and T-LAK cells. While all the data are not shown, we found that several normals' and patients' LAK and T-LAK cultures were totally ineffective, and some had only intermediate levels of ability to induce cytolysis of any of the continuous or primary cell lines. The lack of cytolytic activity in patients' T-LAK cells was not due to prior treatment with chemotherapy and it was also observed in normal donors. There is no clear explanation for the differences in the functional ability of the two separate cultures of effector cells initiated from one of the normal donors (M.F.). Both the

LAK and T-LAK in the initial culture were deficient in all cell lytic activity, even after repeated weekly testing over a 5 month period, whereas a second set of PBM cells established in the subsequent culture, one month later, were cytolytically competent.

Thus, the cells in these non-lytically active cultures were capable of dividing in response to coculture with IL-2, but were unable to express cytolytic activity. This was particularly interesting because further studies showed no detectable phenotypic differences or changes in growth pattern between these and cytolytically active cultures. A similar situation was reported by Jacobs (1986a) in studying LAK cells from glioma patients taken on different days. These data suggest that there can be wide variation in the cytolytic effectiveness of cultured cells intended for use in clinical situations. Studies on the mechanisms of this variation are underway.

T-LAK cells from normal individuals and recurrent glioma patients were able to lyse allogeneic and autologous primary glioma cells in vitro. This is the first data that shows that these effector cell populations are cytolytically active against both allogeneic and autologous glioma cells and are not genetically restricted. The LAK and T-LAK cells from the majority of normal patients also were equally effective in inducing cytolysis of allogeneic glioma cells. In general, glioblastoma cells appear to be quite susceptible to cytolysis induced by competent T-LAK cells in vitro.

Phenotyping studies indicate that the majority of T-LAK cells in these cultures are CD3 T cells. Initially, many of these cells die out and 90–97% of the lymphocytes express T cell markers (CD3) in these cultures after 7 days. The T-LAK cells lack the NK marker CD16, but express the activated T cell marker HLA-DR. While CD3 T cells predominated, there was wide variation in the number of CD4 and CD8 cells in the cultures from each donor. It is surprising that no difference in CD4 or CD8 phenotype was noted between T-LAK cells that were excellent vs. poor killers in vitro. The PAK killers in 2–7 day PHA stimulated human PBL cultures were shown to be predominantly CD3 T cells by Mazumder et al. (1983). Because PHA is a polyclonal T cell stimulator, it is presumed that the T-LAK cells represent the progeny of various T cell subsets and should be heterogenous. Additional double labeling and cell separation studies are ongoing in order to precisely identify the cytolytic cells in the T-LAK cultures.

The present studies show that it is possible to induce, propagate and generate large numbers of T effector cells from peripheral blood of both normal individuals and patients with recurrent glioblastoma. Moreover, these cultures are relatively stable and continue to express cytolytic activity throughout the culture period. This technique provides relatively simple methods for large-scale production of non-restricted CD3 cytolytic T cells which can be employed in adoptive cellular immunotherapies in patients with cancer.

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