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Natural Cell-Mediated Cytotoxicity in vitro and Inhibition of Tumor Growth in vivo by Murine Lymphoid Cells Cultured with T Cell Growth Factor (TCGF)

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Summary. *Lymphoid cells obtained from the spleen, thymus, bone marrow, peripheral blood, and peritoneal exudate of normal mice (BALB/c, BALB/c nude, C57BL/6, C3H) and from spleens of mice bearing a transplantable lung carcinoma or primary mammary carcinoma were expanded in culture for 1–9 months, with an increase in cell number of 10^5- to 10^6-fold per month, in crude or lectin-depleted medium containing T cell growth factor (TCGF). All these cultured lymphoid cell (CLC) lines exhibited strong cytotoxic activity in vitro (assessed by ^{51}Cr-release assays) toward a variety of freshly harvested and cultured syngeneic, allogeneic, and xenogeneic tumor target cells, both lymphoid and solid (including metastatic growths) in origin. Extensive killing was observed against tumor targets that were resistant to lysis by natural killer (NK) cells as well as to NK-sensitive tumor lines. Low levels of cytotoxic reactivity were also demonstrated against fresh and cultured normal lymphoid cells. The CLC had some characteristics of NK cells but also expressed some typical T cell markers. In local Winn-type neutralization assays, CLC delayed or completely inhibited the growth of lymphomas and carcinomas in syngeneic and allogeneic recipients. In mice with metastatic growth of a second-generation transplant of mammary carcinoma, CLC were shown to have some therapeutic effect when administered IV 1 day after cyclophosphamide. No significant beneficial action of IV administered CLC was observed in the absence of chemotherapy in mice implanted with a lung carcinoma. The possibilities of employing TCGF-propagated cytotoxic effector cells in adoptive immunotherapy of human malignancies are discussed.*

Introduction

The discovery of T cell growth factor (TCGF, or interleukin-2) and its use for in vitro cultivation of T cells [23] have provided the opportunity to produce large numbers of lymphoid cells with selected immunological reactivity. Over the past 4 years several groups have been successful in establishing lines and monoclonal cultures of specifically sensitized cytotoxic T lymphoid cells of mouse and human origin [4, 14, 18, 20, 25, 32, 34, 39]. Such cells can be propagated in continuous cultures for periods ranging from several weeks up to several years, with some retention of the original cytotoxic capacity. Murine CLC derived from specifically tumor-sensitized T cells have been shown to be capable, under certain conditions, of retarding or completely inhibiting in vitro growth of mouse tumors [2, 6, 21]. It has also been reported recently that murine and human lymphocytes harvested from normal and tumor-bearing hosts and propagated in TCGF, without any previous in vitro stimulation with tumor cells, frequently display a high level of cytotoxic reactivity against various fresh and cultured tumor cells [1, 12, 16, 30, 38]. Our recent studies and those of other groups indicated that such CLC may possess characteristics of NK cells [3, 12, 16, 24]. Although the cytotoxicity of these cultured effector cells has not ben restricted to a particular type of tumor cell, their potent in vitro reactivity suggests that such an approach might in the future be applied for immunotherapy of human cancer. In this respect, it has been shown recently that TCGF-expanded lymphocytes of cancer patients are cytotoxic in vitro for freshly harvested autochthonous tumor cells [12, 19, 37, 39].

In the present investigation, we examined the characteristics of mouse CLC derived from lymphoid cells of normal and tumor-bearing mice, and assessed their cytotoxic reactivity against a variety of lymphoid and solid tumors. In addition, these CLC were employed in tumor neutralization and immunotherapy assays. The findings reported herein suggest that propagation of lymphoid cells in TCGF selects for, and probably also activates, cells that have a broad range of antitumor cytotoxic activity in vitro and also have some therapeutic activity in vivo.

Materials and Methods

Mice. BALB/c, BALB/c nude, CBA, C3H/HeN MTV$^+$, A/J, and C57BL/6 mice of both sexes and 8–12 weeks old were obtained from Jackson Laboratories, Bar Harbor, Maine, USA, and from Charles River Breeding Laboratory, Stone Ridge, NY, USA.

Tumors. The tumors used in this study are shown in Table 1. Primary, spontaneous mammary tumors were obtained from 10- to 14-month-old C3H/HeN MTV$^+$ mice. Single-cell suspensions of viable tumor cells were obtained from the murine carcinomas (M109, mammary, and 3LL) according to a technique recently described by us [13]. Adherent tumor cell lines were removed from the culture flasks by trypsin/EDTA solution (NIH Media Unit) and washed twice as above. Viability was determined by exclusion of 0.1% trypan blue.

Lymphoid Cells. Lymphoid cells were obtained from normal and tumor-bearing mice. Erythrocytes and dead cells were

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Table 1. Tumor cells employed as target cells in the cytotoxicity assays

Tumor	Carcinogen and origin	Maintenance
M109	Spontaneous lung carcinoma line, BALB/c mice	In vivo, in vitro
Mammary tumor (MT)	Spontaneous mammary carcinoma C3H/HeN MTV$^+$ mice	Primary or 1st passage in vivo; 1st passage in in vitro
3LL	Spontaneous lung carcinoma line, C57BL/6 mince	In vivo
Meth 27A	Methylcholanthrene-induced sarcoma line, BALB/c mice	In vitro
YBA	Moloney virus-induced lymphoma line, CBA mice	In vivo
YAC-1	Moloney virus-induced lymphoma line, A/Sn mice	In vitro
RBL-5	Rauscher virus-induced lymphoma line, C57BL/6 mice	In vivo; in vitro
MBL-2	Moloney virus-induced lymphoma line, C57BL/6 mice	In vivo; in vitro
136.5	Radiation leukemia virus (RadLV)-induced thymoma line, C57BL/6 mice	In vivo; in vitro
PiR-2	Radiation-induced thymoma line, (virus-free) C57BL/6 mice	In vitro
Rl♂1	Radiation-induced lymphoma line, BALB/c mice	In vitro
EL4	Benzpyrene-induced lymphoma line, C57BL/6 mice	In vivo
G-1	Gross virus-induced lymphoma line, W/Fu rats	In vitro
(C58NT)D	Gross virus-induced lymphoma line, W/Fu rats	In vitro
ERTH-V/G	Gross virus-induced lymphoma line, W/Fu rats	In vitro
K562	Erythroleukemia line, human	In vitro
SK MES-1	Lung carcinoma line, human	In vitro
G-11	Breast carcinoma line, human	In vitro
F-265	B lymphoblastoid line, human	In vitro
HT-29	Colon carcinoma line, human	In vitro
MOLT-4	Acute T-cell leukemia line, human	In vitro

removed by centrifugation (400 *g*, 15 min) over a Ficoll-Hypaque layer. Cells were washed twice in 2% FBS-RPMI 1640 and examined for viability in 0.1% trypan blue. For the cytotoxicity assays, lymphoblasts were prepared by cultivating splenocytes (1×10^6/ml) for 3 days with 2 μg concanavalin A/ml (2 × crystallized, Miles-Yeda, Israel) in RPMI 1640 supplemented with 10% FBS, 2 m*M* glutamine, 10 m*M* Hepes buffer, 5×10^{-5} *M* 2-mercaptoethanol, and antibiotics.

Preparation of TCGF-Containing Medium (TCGF-M). The method described by Gillis et al. [5] and by Rosenberg et al. [33] was used, with some modifications. Spleen cells were obtained from 3- to 6-month-old W/Fu rats, or from BALB/c mice, resuspended ($4-7 \times 10^6$/ml) in 5 μg/ml concanavalin A (Con A) in enriched RPMI 1640 medium containing 10% FBS, and incubated for 36–48 h in a 5% CO_2 incubator at 37° C. Cells were pelleted by centrifugation (300 *g*, 10 min), after which the supernatants were collected, spun down (1500 *g*, 20 min), filtered through a 0.22 μ filter, and stored at −70° C up to 6 months or at 4° C up to 2 weeks. This crude preparation was designated C-TCGF-M.

In some experiments, Con A was depleted from the C-TCGF-M by either two sequential incubations for 2 h at 4° C with 15 ml washed, packed Sephadex G-100, or by preparation of proteins precipitated between 45%–75% saturated $(NH_4)_2SO_4$. With both preparations, residual mitogen was neutralized by adding 5–10 mg α-methyl-D-mannoside/ml. These preparations were designated mitogen-depleted TCGF-M (MD-TCGF-M).

The TCGF preparations were tested for their ability to support growth of cultured T cell lines (CLC), whose growth is strictly dependent on TCGF, by measuring ^{3}H-thymidine incorporation into CLC (during a 48-h assay, $5-10 \times 10^4$ CLC in 0.2 ml in flat-bottom microtitration plates showed a stimulation index of 500–1500 with an optimal dose), or by counting the number of CLC 3–4 days after seeding of 2×10^4 cells, in 2 ml, in 24-well cluster plates (No. 3524, Costar, Cambridge, Mass.), where cell number increased 15- to 32-fold with an optimal dose of TCGF.

MD-TCGF-M was shown to have 95%–98% of Con A removed, as assessed by the ^{3}H-thymidine assay of fresh, normal mouse spleen cells, whereas 60%–75% of the original lymphoproliferative activity for CTC was retained. Usually, MD-TCGF-M showed a considerably higher stimulation index than C-TCGF-M when both were tested simultaneously, at optimal concentrations, with CLC in the ^{3}H-thymidine assay.

Establishment of CLC. Lymphoid cells prepared as above were cultivated in enriched RPMI 1640 medium supplemented with 10%–25% C-TCGF-M or MD-TCGF-M. Cultures were initiated with $2-5 \times 10^5$ cells/ml and C-TCGF or with $5-10 \times 10^5$/ml and MD-TCGF and then split, after a thorough pipetting (to detach adherent cells), to 1×10^5/ml every 3–4 days, when cell concentration reached $4-6 \times 10^5$/ml. They were carried out in 25-cm^2, 75-cm^2, or 175 cm^2 tissue flasks (Falcon), in an upright position, with 5–15, 30–60, and 80–200 ml, respectively, of enriched RPMI 1640 medium containing 10%–25% C-TCGF-M or MD-TCGF-M. These CLC were maintained for 1–12 months in 5%–7% CO_2 at 36 ± 0.5° C. Before assaying in the cytotoxicity assays, effector CLC were spun down, incubated for 30 min at 37° C with medium containing 10 mg α-methyl-D-mannoside/ml, and then washed twice with culture medium.

Cytotoxicity Assays. Two to three million normal or tumor target cells were labeled with 200 μCi of ^{51}Cr-sodium chromate in 5% CO_2 at 37° C. Lymphoid normal and tumor target cells were usually labeled in a tube for 2 h, whereas the adherent tumor cells were labeled for 16 h. After labeling, adherent tumor cells were harvested from the flasks following a 1–2 min exposure to prewarmed trypsin-EDTA solution (Gibco). The

labeled cells were washed twice, then 'rested' in medium for 2–3 h at 4° C.

Labeled target cells ($2-10 \times 10^3$, in 0.1 ml) were mixed, in triplicate, with effector lymphocytes (in 0.1 ml) at effector/target cell ratios of 10/1 to 20/1 in 96-well U-shaped Linbro microtitration plates. As controls, target cells were incubated alone. Plates were centrifuged at 300 *g* for 2 min and then incubated in 5% CO_2 at 37° C for either 6 or 18 h. Supernates were harvested with a Titertek supernatant collection system (Flow, Rockwille, Md.). Percent specific ^{51}Cr release was calculated according to the following formula:

$$a - b/c - b \times 100$$

where a is counts per minute (cpm) of experimental group, b is cpm of the control group, and c is the total releasable cpm obtained by incubating the targets in 5% Triton X-100 (giving 80%–92% of the total label incorporated by the cells).

Spontaneous ^{51}Cr release rarely exceeded 25% during the 18-h assay, and data from such experiments were omitted. Each effector/target cell combination was assayed in three to ten separate experiments, and the means of the cytotoxicity values from these experiments are shown.

To assess antibody-dependent cellular cytotoxicity (ADCC), labeled G-1 (see Table 1) target cells were incubated for 6 h with the effector cells in the presence of a 1/400 dilution of a hyperimmune rabbit anti-G-1 antiserum.

In some experiments, effector cells were pretreated with interferon as follows: 2×10^6 cells were incubated in 0.5 ml with 10^4 units of mouse fibroblast interferon (Calbiochem-Behring Corp., La Jolla, Calif.) for 2 h in 5% CO_2 at 37° C, and the cells were then washed twice and incubated for an additional 6 or 18 h with labeled target cells as above.

Analysis of Cell Surface Markers. Cell surface markers were studied by: (a) a rosette technique or (b) a complement-dependent cytotoxicity assay (CDC). Before assay, cells maintained in TCGF were spun down, incubated with 10 mg α-methyl-D-mannoside/ml for 30 min at 37° C, and then washed twice in 5% FBS-RPMI 1640.

Direct rosetting with sheep or ox erythrocytes was performed by mixing 3×10^5 cells with 1×10^7 erythrocytes; the cell mixture was incubated for 10 min at 37° C and then spun at 200 *g* for 5 min. The pelleted cells were stored on ice for 30 min, the cells were resuspended gently, and rosettes (lymphocytes binding three or more erythrocytes) were scored under the microscope. Antibody-coated erythrocytes (EA), for detecting Fc^+ cells, were prepared as above from erythrocytes precoated (for 30 min at 37° C) with a subagglutinating dose (1/100 to 1/200) of of anti-erythrocyte IgG or IgM antibodies (Cappel Laboratories Inc., Cochranville, Pa.).

Antigen expression was determined by a CDC assay, with a monoclonal IgM anti-Thy 1.2 [12], anti-Lyt-2 generously provided by Drs U. Hammerling and Gloria Koo, Sloan Kettering Institute for Cancer Research, New York, NY or arsanilite-conjugated monoclonal anti-Lyt 1 and anti-Lyt 2 (Becton Dickinson, Mountain View, Calif.); or with rabbit anti-asialo GM1 antiserum [10], which was kindly provided by Drs K. Okumura and S. Habu, University of Tokyo. One million cells in 0.1 ml were incubated for 30 min at room temperature with varying dilutions (1/50 to 1/1,000, in 0.1 ml) of the antiserum. For the arsanilated antibodies, cells were then incubated with rabbit anti-arsanilate antibody. Subsequently, cells were suspended in 2 ml medium, spun down, incubated with a 1/6 to 1/9 dilution (0.20 ml) of pretested rabbit complement (Cappel) for 45 min at 37° C, then washed once and examined for viability with 0.1% trypan blue.

Winn Neutralization Assays. Inhibition of tumor growth in vivo was assayed by the following procedure: Control mice received 1×10^5 (in 0.1 ml) viable syngeneic lymphoma or carcinoma tumor cells by SC or IM injection. Experimental mice were inoculated with the same number of tumor cells as had been admixed with 3×10^6 to 1×10^7 of either fresh normal spleen cells or splenocyte-derived syngeneic or allogeneic CLC (0.2 ml) derived from tumor-bearing mice.

Results

Growth Capacity of Various Lymphoid Cell Populations in TCGF-Containing Media

Lymphoid cells obtained from the spleen of normal and tumor-bearing mice were cultured for various periods of time, in different culture vessels, in the presence of crude or mitogen-depleted TCGF of mouse and rat origin. The growth pattern of the various cultures was monitored three times a week by viable cell counting, and the results obtained with some of the splenocyte cultures are shown in Table 2. The following conclusions can be drawn: (a) Rat TCGF was superior to mouse TCGF for all types of cells, especially when cells were cultivated in large culture flasks; (b) Under the conditions employed (Table 2) a greater increase in cell number was attained in Costar plates than in cultures flasks; (c) In cultures initiated and maintained with MD-TCGF-M, the majority of the cells died during the first week (data not shown), after which the few remaining viable cells proliferated almost to the same extent as did cells maintained in C-TCGF-M; (d) Cultures of splenocytes from nude mice (data not shown) and from mice with disseminated carcinoma showed a rather slow growth rate during the first 7–10 days of culture, but thereafter growth was similar to that of cultures originated from normal splenocytes.

In other preliminary experiments, we compared the growth rate, in rat C-TCGF-M, of spleen, thymus, peripheral blood, peritoneal exudate, and bone marrow cells of C57Bl/16 and BALB/c mice. It was found that lymphoid cells of spleen, peripheral blood, and peritoneal exudate had a similar growth rate during the period studied (2–3 months), whereas thymocytes showed a slightly lower proliferative capacity. In contrast, only a small minority of the bone marrow cells survived after the first week; the remaining cells proliferated at a much lower rate, and cultures died after 4–6 weeks.

In the course of the present studies, over 20 culture lines of splenocytes from various strains of mice were initiated and maintained for periods of 3–12 months. Some, but not all, cultures underwent a 'crisis' phase at 3–6 weeks after initiation. During that period cell viability dropped sharply and morphologic changes (vacuolization, granulation, and enlargement of the cells) were noticed. Cultures could be rescued by frequent removal of dead cells (by centrifugation over Ficoll-Hypaque at 400 *g* for 15 min) and medium changing, and by transferring the cells temporarily to Costar plates or by incubating the culture flasks in a tilted position. In addition, cultures could be improved by introducing irradiated (3,000 rads) syngeneic spleen cells at $1-5 \times 10^5$/ml, weekly, during the crisis period.

Table 2. Growth capacity of splenocytes from normal and M109 tumor-bearing BALB/c mice in crude and mitogen-depleted mouse and rat TCGF-containing media

Donors of splenocytes	Source of TCGF[a]	Magnitude of increase in cell number at day 15				
		Costar plate[b]	50 ml flask[c]			
Normal	Mouse	586	313			
	Rat	3600	1296			
Tumor-bearers	Mouse	202	121			
	Rat	1446	724			
		Magnitude of increase in cell number at:[d]				
		Day 10	Day 30	Day 60	Day 90	Day 180
Tumor-bearers	Rat, crude	256	4.5×10^5	2.4×10^{10}	1.5×10^{10}	2.4×10^{23}
	Rat, mitogen-depleted	64	5.7×10^3	3.7×10^9	3.3×10^{13}	1.5×10^{22}

[a] Used at 25% v/v
[b] Cultures started with 3×10^4 cells/ml and split to 1×10^4/ml every 3–5 days
[c] Cultures started with $3–5 \times 10^5$ cells/ml and split to 1×10^5/ml
[d] Cultures maintained in 250 ml flasks and split as in [c]
Results are means of three experiments. All flasks were incubated upright

Under the conditions employed, cultures maintained in C-TCGF-M expanded at a rate of 10^5- to 10^6-fold per month with generation time of 16–24 h.

Cytotoxic Activity of the CLC

The results of a large number of experiments performed with a wide range of normal and tumor target cells are summarized in Tables 3–5. When fresh noncultured splenocytes from normal or tumor-bearing mice were used only the classic NK-sensitive lymphoma cells (YAC-1, RL♂1) were killed to an appreciable extent, whereas all other lymphoid and solid tumor cells were resistant to lysis. As was found previously in our laboratory [8], fresh splenocytes from mice with advanced tumors had lower cytotoxic activity than splenocytes of normal mice (Tables 3 and 4). Splenocytes cultured for 6 days in enriched RPMI 1640 medium (with 10% FBS) in the absence of TCGF exhibited a slightly lower reactivity toward YAC-1 and RL♂1 and increased cytotoxicity (15%–25% in an 18-h assay) against several cultured solid tumor cells (e.g., M109, MT) (data not shown).

All the cultures maintained by the periodic addition of TCGF showed a strong cytotoxic activity against most of the tumor target cells tested (Tables 3 and 4): fresh and cultured, syngeneic and allogeneic, lymphoid and solid, NK-sensitive and NK-resistant. Cultured M109 and MT were slightly more sensitive to killing, in general, than freshly harvested transplanted M109 or primary spontaneous MT. With most of the CLC, maximum cytotoxic activity was observed after 2–3 weeks in culture. The magnitude of the cytotoxicity against various targets thereafter did not change appreciably during the first 3–4 months of culture, and a strong cytotoxic effect was noticed even at an effector/target ratio of 1/1 (data not shown). Later on, however, some lines showed a gradual diminution in cytotoxicity, particularly against the NK-insensitive solid tumor cells. CLC maintained with MD-TCGF-M and with C-TCGF-M, and those obtained from either normal or tumor-bearing mice, showed a similar pattern and magnitude of cytotoxicity against most targets tested.

Preliminary experiments have indicated that CLC originating from spleen, peripheral blood, and peritoneal exudate all killed a wide range of lymphoid and solid tumor targets. In contrast, TCGF-cultured bone marrow cells were mainly effective against NK-sensitive lymphoma target cells and cultured thymocytes were more cytolytic to NK-resistant solid tumor cells. Additional experiments are under way to ascertain whether these organs have different subsets of NK precursor cells.

Low but significant cytotoxicity by CLC was demonstrated with xenogeneic rat and human cultured tumor cells (Tables 3 and 4), which are rather resistant to lysis by fresh NK effector cells.

A considerable level of cytotoxicity was also found against some normal murine lymphoid target cells (Table 5), especially when the ^{51}Cr assay was extended to 16–18 h. Con A-induced allogeneic lymphoblasts were more susceptible than syngeneic lymphoblasts, and among the freshly harvested normal lymphoid cells, thymus cells and adherent peritoneal exudate cells were generally more sensitive than the other cell types, while spleen cells were quite resistant.

In other experiments, we found that BALB/c CLC were highly cytotoxic to M109 tumor cells derived from metastatic foci in the lung, spleen, adrenal, and liver (data not shown). The cytotoxic activity against all the tumor targets shown in Tables 3 and 4 was augmented appreciably (by 30%–70%) following a 2-h pretreatment at 37° C of the effector CLC with 10^4 units of mouse fibroblast interferon [12].

Another function associated with NK cells is ADCC activity [17, 27, 36]. As part of the characterization of the CLC, it was therefore of interest to evaluate their ADCC activity. The data presented in Table 6 show that BALB/c and C3H CLC can function as effector cells in an ADCC system, against antibody-coated target cells, in addition to their ability to lyse target cells directly. The ADCC activity, but not the direct cytotoxicity, could be blocked almost completely when protein A (previously shown to selectively inhibit ADCC [11]) was added to the assay.

Table 3. Cytotoxic activity of fresh and cultured lymphoid cells from normal and M109 tumor-bearing (T.B.) BALB/c mice against mouse, rat, and human tumor target cells

Target cells	Mean percent cytotoxicity[a] with								
	Normal spleen	Cultured normal spleen[b]	Cultured normal spleen[c]	Spleen from T.B.	Cultured spleen from T.B.[b]	Cultured spleen from T.B.[c]	Cultured bone marrow[b]	Normal spleen from nudes	Cultured spleen from nudes[b]
YAC-1	32/55[d]	57/82	55/77	8/15	60/88	63/94	–	32/48	59/90
RL♂1	28/49	45/87	42/89	13/25	36/81	34/80	39/80	–	55/94
M109[e]	2/4	45/77	51/80	1/3	60/92	53/91	5/18	2/7	28/53
MT[d]	2/6	21/39	27/43	1/2	17/41	20/48	–	1/6	–
3LL	3/5	14/28	10/21	3/7	15/31	12/26	–	–	11/23
Meth 27A	–[f]	–	–	–	14/24	18/31	–	–	–
RBL-5[e]	–	–	–	3/8	33/77	44/82	–	–	–
EL4[e]	2/8	21/51	16/39	2/7	41/80	35/79	7/29	–	26/55
MBL-2	4/7	34/71	36/80	3/7	36/77	39/84	–	–	–
PiR-2	1/4	16/35	–	–	–	–	–	–	–
G-1	3/5	–	–	–	6/14	7/21	–	–	–
(C58NT)D	–	–	–	–	22/39	14/26	–	–	–
ERTH-VG	–	–	–	–	–	–	–	–	25/61
K562	2/6	6/14	–	–	10/19	8/18	–	–	14/23
SK-MES 1	–	–	–	–	6/16	5/14	–	–	6/16
G-11	1/4	–	–	–	7/16	12/24	–	–	3/8
F-265	–	–	–	–	20/45	23/49	–	–	–
HT-29	–	–	–	–	1/5	2/6	–	–	3/8
MOLT-4	1/5	–	–	–	6/13	–	–	–	–

[a] Means of two to eight determinations in separate experiments for each effector/target combination. Effector LCL obtained 1–6 months after culture initiation with TCGF. Effector/target cell ratio 10–20/1
[b] Cultures maintained with C-TCGF-M
[c] Cultures maintained with MD-TCGF-M
[d] First value represents data from 6-h assay and second from 18-h assay
[e] Freshly harvested in vivo-passaged tumor cells
[f] Not tested

Table 4. Cytotoxic activity of fresh and cultured lymphoid cells from normal C57BL/6 and mammary tumor-bearing (T.B.) C3H mice against mouse, rat, and human tumor target cells

Target cells	Mean percent cytotoxicity[a] with					
	C3H spleen from T.B.	Cultured C3H spleen from T.B.[b]	Cultured C3H spleen from T.B.[c]	C57BL/6 spleen	Cultured C57BL/6 spleen[b]	Cultured C57BL/6 thymus[b]
YAC-1	23/36[6]	46/94	59/94	25/42	43/87	–
RL♂1	16/24	40/85	39/66	19/37	54/90	19/46
M109[e]	1/2	37/82	58/94	2/5	28/67	24/78
MT[d]	2/4	25/44	24/49	1/3	–	–
Meth 27A	–	7/18	6/14	–	–	–
EL4[d]	–	–	–	1/3	14/33	10/26
MBL-2[e]	–	38/67	30/64	–	–	–
YBA[e]	–	13/28	–	1/4	36/69	–
136.5	–	29/65	–	1/3	50/83	–
PiR-2	–	–	–	0/2	21/46	–
G-1	–	6/18	13/25	–	–	–
K562	–	11/27	9/24	–	–	–
SK-MES 1	–	5/11	9/18	–	–	–
G-11	–	10/19	7/13	–	–	–
F-265	–	16/38	19/40	–	–	–
HT-29	–	4/9	2/4	–	–	–
MOLT-4	–	4/7	–	–	–	–

[a–f] See legends to Table 3

Table 5. Cytotoxic activity of CLC against normal lymphoid target cells

Effector CLC[a]	Mean % cytotoxicity (E/T = 20/1)[b]					
	Fresh BALB/c spleen	Fresh BALB/c bone marrow	Fresh BALB/c thymus	Fresh BALB/c peritoneal exudate	BALB/c blasts	C3H blasts
N. BALB/c MD-TCGF-M	–	–	–	–[c]	3/11[d]	8/22
T.B. BALB/c C-TCGF-M	1/5	4/7	8/17	7/14	7/14	14/28
T.B. BALB/c MD-TCG-M	0/3	0/2	5/9	12/23	5/12	19/39
T.B. C3H C-TCGF-M	3/6	4/5	5/14	5/11	11/39	3/18
T.B. C3H MD-TCGF-M	1/4	5/10	6/12	7/12	17/48	6/25

[a] CLC obtained 1–3 months after culture initiation from normal (N.) or M109 and mammary tumor-bearing (T.B.) BALB/c and C3H mice
[b] Means of three separate experiments
[c] Not tested
[d] 6 h assay/18 h assay

Table 6. ADCC Activity of BALB/c and C3H CLC

Effector CLC[a]	% Cytotoxicity[b] at			
	6 h (E/T = 40/1)		18 h (E/T = 4/1)	
	G-1	G-1 + Ab	G-1	G-1 + Ab
1. BALB/c	4.6	15.9	2.2	30.8
2. BALB/c	2.2	11.3	1.8	34.1
3. C3H	1.9	22.7	5.5	29.6
4. C3H	–	–	7.2	45.9

[a] CLC derived from splenocytes of M109 tumor-bearing BALB/c mice (1, 2) and mammary tumor-bearing C3H mice (3, 4) propagated with either crude TCGF (1, 3) or Con A-depleted TCGF (2, 4)
[b] Means of three separate experiments

Surface Markers of the CLC

To further characterize our cultured cells, we assessed several surface markers that have been associated with either cytotoxic T lymphocytes or NK cells. The results shown in Table 7 indicate that: (a) virtually all BALB/c CLC were Thy 1.2^+ and asialo $GM1^+$, as indicated by cell killing with specific antisera and complement and by the parallel loss in cytotoxic activity; (b) a considerable portion of the cells expressed Lyt 2, a smaller proportion expressed Lyt 1, and a portion of the effector cells appeared to possess neither of these markers; and (c) on rosetting with IgG-coated ox erythrocytes only a small minority demonstrated an Fc receptor, whereas, surprisingly, a substantial proportion of the CLC formed spontaneous rosettes with sheep erythrocytes. No rosetting with IgM-coated ox erythrocytes was detected.

Killing of CLC with anti-Thy 1.2 and complement required higher amounts of these reagents than were needed to lyse fresh T cells from the spleen and thymus. Whereas a 1/2,000 dilution and a 1/12 dilution of the antiserum and complement, respectively, were effective with the fresh T cells, 1/200 and 1/6 dilutions, respectively, were needed to lyse ≥ 80% of the CLC. This difference suggests that Thy 1.2 is present on the CLC at a lower density or is less accessible to antibody + complement attack than on fresh T cells.

Antitumor Activity of CLC in vivo

In light of the potent antitumor activity demonstrated by the CLC in vitro, it was interesting to determine whether they could also affect tumor growth in vivo.

In Winn neutralization assays (Table 8), BALB/c CLC afforded partial protection against a syngeneic lymphoma (RL♂1) and carcinoma (M109) in BALB/c mice and against an allogeneic lymphoma (MBL-2) in C57BL/6 mice. When sufficient CLC were given (i.e., at a ratio of CLC/tumor ≥ 30/1), a number of mice in the experimental groups remained tumor-free and, moreover, the remaining mice developed the tumor later and showed a longer survival time than did the control mice. C3H CLC at a ratio of 100/1 completely inhibited mammary tumor growth and mice remained tumor-free for more than 4 months. In contrast to CLC, fresh BALB/c splenocytes were totally ineffective against M109 (Table 8, expt 2).

Mice that survived tumor-free for 3 months following inoculation of MBL-2 or M109 tumors plus CLC were challenged SC with 1×10^5 of the corresponding tumor cells. Whereas three of four mice that received implants of MBL-2 did not develop tumors during 3 months, six of eight mice that received M109 by injection developed tumors and died. In the latter case, the time of tumor appearance and death were significantly delayed compared with control mice. These findings suggest that exposure of naive mice to CLC and tumor cells may be capable of mobilizing the host's immune responsiveness to some extent. No experiments were performed to test whether this partial protection was tumor-specific.

Table 7. Surface markers of BALB/c CLC

Assay[a]	% Rosettes	Results[b]		
		% Cells killed	% Inhibition of cytotoxicity[c] against	
			YAC-1	M109
SRBC rosettes	12–25			
SRBC-IgG rosettes	15–30			
Ox RBC rosettes	0			
Ox RBC-IgG rosettes	0–4			
Ox RBC-IgM rosettes	0			
Monoclonal anti-Thy 1.2+C′		84–95	69– 89	73–98
Monoclonal anti-Lyt 1+C′		10–32	5– 14	15–33
Monoclonal anti-Lyt 2+C′		40–75	28– 40	37–70
Anti-asialo GM1+C′		88–94	94–100	89–95

[a] Rosettes were performed at a 33/1 RBC/CLC ratio. The monoclonal antibodies were used at a final dilution of 1/200. For more details, see *Materials and Methods*

[b] Values indicate the range of three experiments

[c] As determined by the ^{51}Cr assay at a 10/1 effector/target cell ratio. Percent inhibition calculated in relation to percent cytotoxicity with effector CLC treated with antiserum alone or with normal mouse serum plus complement. In other experiments, addition during the cytotoxicity assay of anti-Lyt 2, but not anti-Lyt 1, in the absence of complement, caused up to 50% inhibition of cytotoxicity (data not shown)

Table 8. Tumor neutralization (Winn) assays in vivo with mouse CLC

Expt. no.	Tumor[a]	Source of CLC[b]	CLC/tumor ratio in inoculum	Median survival time (days)	Tumor-free survivors/total (day 120)
1	M109	None	–	32	0/8
		N. BALB/c C-TCGF-M	30/1	37	0/12
		T.B. BALB/c C-TCGF-M	30/1	48	0/14
	RL♂1	None	–	29	0/8
		T.B. BALB/c	30/1	54	1/8
	MBL-2	None	–	23	0/8
		T.B. BALB/c C-TCGF-M	30/1	77	3/8
2	M109	None	–	36	0/8
		N. BALB/c fresh spleen	100/1	39	0/8
		T.B. BALB/c C-TCGF-M	100/1	58	3/8
		T.B. BALB/c MD-TCGF-M	100/1	>120	6/8[c]
	MBL-2	None	–	24	0/8
		T.B. BALB/c C-TCGF-M	100/1	68	5/8[c]
3	Mammary tumor	None	–	77	1/10
		T.B. C3H C-TCGF-M	70/1	>120	10/10[c]

[a] 1×10^5 tumor cells were injected SC in the corresponding syngeneic normal mice

[b] CLC were derived from normal (N) BALB/c mice, from M109 tumor-bearing (T.B.) BALB/c mice and from mammary tumor-bearing C3H mice, with crude (CTGF-M) or Con A-depleted (MD-TCGF-M) conditioned medium. CLC were obtained at day 15 (expt. 1), day 30 (expt. 2), and day 60 (expt. 3) after culture initiation with TCGF. In expt. 2 fresh normal splenocytes were used

[c] Significantly different ($P < 0.05$) by chi-square analysis from the control group

Table 9. Effect of CLC on lung metastases[a] from a transplanted M109 lung

	Number of metastases in the lung	
	Mice receiving tumor only	Mice receiving tumor + CLC
	10	2
	12	6
	20	9
	24	13
	24	19
	31	22
	41	24
	41	32
	48	35
	–	55
Mean ± SEM	27.9 ± 4	21.7 ± 4

[a] BALB/c mice received 1.5×10^5 fresh M109 tumor cells IV alone or tumor cells followed 3 days later by 7×10^6 BALB/c CLC administered IV. Lung metastases were counted 4 weeks after tumor implantation. Similar results were obtained when the same number of CLC was administered IV simultaneously with the tumor cells or 3 h later (data not shown)

Table 10. Chemoimmunotherapy of mammary tumor in C3H mice with cyclophosphamide and CLC

Treatment[a]	No. of mice survived[b]/ total no. of mice	Mean survival time ± SEM[d] (in days)
Tumor only	1/14	64 ± 4
Tumor + cyclophosphamide	7/15[c]	82 ± 7[e]
Tumor + cyclophosphamide + CLC	10/16[c]	105 ± 5[e,f]

[a] C3H mice were injected IV with 2.5×10^5 freshly harvested mammary tumor cells (after one passage in vivo). Cyclophosphamide (180 mg/kg) was administered IP on day 7. TCGF-propagated splenocytes derived from mammary tumor-bearing C3H mice were injected (15×10^6 per mouse) IV on day 8

[b] Experiment terminated on day 150

[c] Significantly different from control group ($P < 0.05$ by chi-square analysis), but not different from each other

[d] Of mice that died with a tumor

[e] Significantly different from control group ($P < 0.05$ by Student's *t*-test)

[f] Significantly different from chemotherapy group ($P < 0.05$)

Systemic administration of CLC to mice with established disseminated tumors showed a limited therapeutic effect in the two experiments performed thus far. In the first, CLC given to BALB/c mice that had received M109 lung carcinoma by injection 3 days previously reduced (mean 22%) the number of lung metastases slightly but not significantly (Table 9). In the second experiment, C3H mice carrying a transplanted mammary tumor were treated with either cyclophosphamide alone or cyclophosphamide and CLC (Table 10). The proportions of mice surviving in the chemotherapy-treated group and in the chemoimmunotherapy-treated group were similar. However, the survival time of mice that died of tumor in the latter group was approximately 20% (significant, $P < 0.05$) greater than that of the chemotherapy alone group.

Because of their cytotoxic activity in vitro against some normal target cells, experiments were also carried out to evaluate the effect of inoculation of large numbers of CLC into immunodepressed recipients. In those experiments, mice pretreated with 200 mg cyclophosphamide/kg received 15×10^6 syngeneic CLC by IV injection once, or 10×10^6 CLC twice 7 days apart. The treated mice did not show any clinical symptoms of graft-versus-host disease.

Discussion

The main findings of the present studies are: (a) Lymphoid cells from normal mice and from mice bearing large metastatic carcinomas could be expanded in culture to very large numbers within a short period of time, whether crude or mitogen-depleted TCGF preparations were used; (b) Although no specific tumor stimulation was employed, the propagated lymphoid cells expressed a wide spectrum of cytotoxic reactivity against NK-sensitive and NK-resistant lymphoid and solid tumor cells, both fresh and cultured and of both syngeneic and allogeneic origin; (c) Under the conditions employed, the cultured cells were capable of inhibiting tumor growth in a local Winn neutralization assay but were relatively ineffective when administered systemically into hosts with established disease.

Of primary interest is the nature of the cytotoxic effector cells among the CLC. It has previously been demonstrated, in studies of CLC initiated from peripheral blood mononuclear leukocytes of normal human donors, that several types of cytotoxicity were detectable [29]: NK-like activity, ADCC, lectin-induced cytotoxicity, and polyclonally activated cytotoxicity against allogeneic target cells. The present results indicate a similar pattern. The relatively strong reactivity of CLC against allogeneic normal blast cells is suggestive of polyclonally activated cytotoxic T cells. However, the observations of cytotoxicity also against syngeneic lymphoblasts and tumor cell lines, and against a xenogeneic target cell like K562, which has been shown to lack expression of major histocompatibility antigens [14], indicate that other types of cytotoxic effector cells exist in the cultures. It seems likely that a major contribution to the cytotoxicity is by cells with the characteristics of NK cells. The cytotoxic reactivity of CLC against a wide array of syngeneic, allogeneic, and xenogeneic target cells is reminiscent of a similar broad reactivity of NK cells. YAC-1 and RLδ1, which are widely used as sensitive target cells for murine NK cells, were both highly susceptible to lysis by the CLC. The reactivity against some normal thymus cells, bone marrow cells, and peritoneal exudate cells, but not against normal spleen cells, has also been observed for fresh NK cells [26]. However, in addition to cytotoxicity against NK-susceptible targets, the CLC were also found to have considerable reactivity against several solid tumor cell lines, both transplantable and spontaneous, and other targets that are usually found to be resistant to NK cells. It is possible that the NK cells in the cultures are more highly activated than those found normally in the spleen and that such reactivity, particularly in a long-term assay, can result in lysis of relatively insensitive targets. This possibility is supported by previous findings that M109, which is entirely resistant to NK activity in a 4-h in vitro cytotoxicity assay, could be shown to be susceptible in longer-term in vitro assays and in an in vivo assay of NK activity [31]. Alternatively, or in addition, other types of natural effector cells, e.g., NC cells [35], which have been shown to have a predilection for reactivity against NK-resistant solid tumor target cells, might be involved. The findings that

the cytotoxic reactivity of CLC could be appreciably augmented by pretreatment with interferon are most compatible with an involvement of NK cells and possibly NC cells, since such effector cells but not cytotoxic immune T cells [9] have been found to be consistently augmented by interferon. Furthermore, the finding of ADCC activity by the CLC is highly indicative of the presence of NK cells, since NK cells have ben shown to be the main type of nonadherent lymphoid cells mediating ADCC [17, 27, 36]. With regard to the surface markers on the CLC, and particularly on the cytotoxic cells, the findings of strong reactivity with anti-Thy 1 and anti-asialo GM1, and lower reactivity with anti-Lyt 1, are quite compatible with involvement of NK cells. Such reactivity would be unusual for freshly harvested NC cells, since they have been found to be quite resistant to complement-dependent lysis by these antibodies. The observations that monoclonal antibodies to Lyt 2 reacted with a considerable portion of the CLC and reduced their cytotoxic activity are quite surprising since this antigen has usually not been detected on fresh NK cells [15]. However, Minato et al. [22] recently reported that Lyt 2 was present in a subpopulation of NK cells. In addition, it is quite possible that the phenotype of NK cells changes considerably in culture, acquiring more markers typical of T cells. Such observations have been made when purified populations of human NK cells have been cultured in the presence of TCGF [28]. On the basis of the present results, it is not possible to determine whether all the observed cytotoxic activities are mediated by the same effector cells or whether a variety of different effectors are present in CLC. Evaluation of cloned populations of CLC is needed to answer this question, and in other recent studies [12] we have found that our lines are composed of at least two distinct types of clones with the characteristics of NK cells, one class being highly effective only against NK-sensitive murine target cells and the other being highly cytotoxic against NK-resistant solid tumor cells as well against NK-sensitive targets. Of interest was the observation that some clones of both types were positive for Lyt 2 as well as for Thy 1, asialo GM1 and the NK2 alloantigen.

A striking finding in these studies was that all the CLC lines, whether initiated from either normal or tumor-bearing mice, had considerable cytotoxic activity. This would imply that cells with cytolytic capacity preferentially survice and grow under the culture conditions used here. It is not yet clear whether this is due to propagation of initially present NK cells or whether some T cells or other cells differentiate in culture into cytotoxic effector cells. Both interferon and TCGF have been shown to be able to boost NK activity and/or promote the differentiation of NK cells from their precursors [7, 22].

The ability of the CLC to kill in vitro fresh syngeneic tumor cells and to affect tumor growth in vivo without any previous intentional tumor stimulation may have practical implications. By this approach, lymphoid cells from cancer patients could be expanded direxty with TCGF and subsequently administered in large quantities to the autologous donors. Our recent studies with TCGF-cultured lymphocytes from patients with various carcinomas have already shown an appreciable cytotoxic reactivity in vitro toward autochthonous and allogeneic fresh tumor cells. Such cells have also been shown to inhibit human tumor growth in athymic nude mice [12]. Despite the many difficulties foreseen in using this approach, the great potential for successful clinical application of TCGF-propagated antitumor effector cells should encourage further investigations aimed at a better understanding of the in vivo behavior of such cells.

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