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Antitumor Reactivity *in Vitro* and *in Vivo* of Lymphocytes from Normal Donors and Cancer Patients Propagated in Culture with T Cell Growth Factor (TCGF)*

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Abstract—*Peripheral blood lymphocytes (PBL) from* 38 *normal donors and from* 27 *cancer patients were propagated in bulk cultures for* 3–6 weeks *using T cell growth factor (TCGF). In addition, cultures derived from lymphocyte preparations enriched for or depleted of natural killer (NK) cells and several clones of cultured cells were studied. The following main observations were made: (a) PBL of both patients and healthy donors could be expanded to large numbers (up to 2500-fold); (b) CLC derived from unfractionated PBL exhibited intermediate levels of cytotoxic activity against autologous and allogeneic fresh lung tumor cells and strong cytotoxicity toward several cultured adherent tumor cells; (c) whereas cultures originated from populations enriched for NK cells were highly cytotoxic against both adherent tumor target cells and against an NK-sensitive leukemic cell line (K562), cultures derived from populations depleted of NK cells were preferentially cytotoxic to adherent target cells; (d) clones of CLC were also strongly cytotoxic, but 2 out of 3 clones tested showed a narrower spectrum of target cytotoxicity than that of uncloned CLC; (e) CLC, when mixed with two carcinoma cell lines, were able to inhibit tumor growth in nude mice.*

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Abbreviations: FBS, fetal bovine serum; LDCC, lectin-dependent cellular cytotoxicity; LGL, large granular lymphocytes; MLTC, mixed lymphocyte tumor cell culture; NK, natural killing; PBS, phosphate-buffered saline; SRBC, sheep red blood cell; TCGF, T cell growth factor (also known as interleukin-2).

INTRODUCTION

THE DISCOVERY of T cell growth factor (TCGF or interleukin-2) [1, 2] in supernatants of lymphoid cells stimulated with lectins or antigens has provided the possibility of producing large quantities, and even monoclonal lines, of T cells with cytotoxic, helper or suppressor activity [3, 4]. Major efforts have been made over the last 3 yr, with both human and murine cells, to develop cytotoxic lymphoid cell lines with antitumor reactivity in the hope of applying such reagents in adoptive immunotherapy of cancer. T cells specifically sensitized to normal alloantigens or to tumor antigens and subsequently expanded with TCGF have been shown to maintain their specific cytotoxic reactivity to some degree over a long period of time [5–14], and recent studies in mice have already indicated that TCGF-propagated cytotoxic cells can also exert some therapeutic effects *in vivo* [15–17].

Since tumor-bearing individuals might be expected to have T cells sensitized *in vivo* to tumor-associated antigens, it was expected that such cells could be selectively expanded with TCGF without the need for further *in vitro* stimulation. In this respect it has been reported recently that murine and human lymphocytes harvested from tumor-bearing hosts and propagated in TCGF frequently display a high level of cytotoxic reactivity against various fresh and cultured tumor cells [18–20], including freshly harvested autochthonous tumor cells [10, 11, 21–23].

However, it has also been found that *in vitro* incubation of lymphoid cells from normal individuals with TCGF could also lead to the propagation of cultured lymphoid cells (CLC) with substantial cytotoxic reactivity against various tumor cells [7, 8, 18, 24–28] and that such CLC may possess characteristics of NK cells rather than specifically immune T cells. Such observations indicate the need to carefully analyze the characteristics of CLC with antitumor reactivity and to determine the specificity of their cytotoxicity.

In this study we have initiated CLC from both cancer patients and normal donors. Their characteristics have been examined and their cytotoxic reactivity assessed against a variety of lymphoid and solid tumor cells and also against various normal target cells. The results indicate that CLC derived from tumor-bearing and normal individuals have very similar features, and that the cytotoxic cells appear to be mainly NK cells and possibly other natural effector cells or *in vitro*-activated lymphoid cells. Although the cytotoxicity of these CLC has not been restricted to a particular type of tumor cell, the ability to grow large numbers of cells with potent *in vitro* reactivity suggests that such an approach might in the future be applied for immunotherapy of experimental and human tumors. In support of this possibility, we have found that human CLC have some *in vivo* antitumor activity.

MATERIALS AND METHODS

Peripheral blood lymphocytes (PBL)

Heparinized blood was obtained from 27 cancer patients with lung (16), breast (9) and colon (2) carcinoma (males and females, 27–73 yr of age, mean 55 yr) that had not been treated with chemotherapy or radiation therapy for at least 3 months before blood drawing and from 38 normal donors (males and females, 25–58 yr of age, mean 32 yr). The mononuclear cells were isolated by Ficoll-Isopaque gradient centrifugation [29]. For Percoll gradient fractionation (see below) leukocyte-enriched buffy coats were obtained from the plateletpheresis of 300 ml blood, and PBL were isolated as above. The separated cells were washed twice in RPMI 1640 medium. For some experiments PBL (1×10^6/ml) were stimulated for 3 days with 5 μg/ml concanavalin A and used as target cells.

Percoll gradient fractionation

PBL were separated by centrifugation over a discontinuous (7-layer) density gradient of Percoll following removal of adherent cells (monocytes and B lymphocytes) by plastic adherence and nylon wool column, as described recently [30, 31]. Large granular lymphocytes (LGL) of low density, shown previously to possess most of the NK activity in fresh human PBL [31], were collected (70–80% purity) from the top 2nd and 3rd Percoll fractions. T cells (see Results) of high density, containing less than 2% LGL, were harvested from the 6th and 7th layers. The separated cells were washed twice with RPMI 1640 containing 5% human AB serum.

Human tumors

Tumor biopsies (1–3 g) excised from patients with squamous cell carcinoma and adenocarcinoma of the lung were received 4–8 hr after surgery. They were stored on ice in RPMI 1640 medium with antibiotics until processing. The following tissue culture lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 3 mM glutamine, 10 mM HEPES buffer and 50 μg/ml gentamicin: SK-MES1, CaLu and 9812, derived from patients with lung carcinoma; HT-29, derived from a colon carcinoma; MCF 7 and G-11, derived from patients with breast carcinoma; and

K562, derived from a patient with chronic myeloid leukemia in blast crisis.

Mouse tumors

Madison 109 (M109), a spontaneous lung carcinoma of BALB/c mice [32], was maintained *in vivo* by biweekly serial passages of $2-5 \times 10^5$ cells subcutaneously or intramuscularly. Single-cell suspensions were prepared from tumors of 1.0–1.5 cm diameter (see below). YAC-1, a Moloney virus-induced lymphoma of A/Sn mice [33], and RL♂1, a radiation-induced lymphoma of BALB/c origin, were maintained as culture lines in the same medium used for cultures of human tumors.

Isolation of tumor cells from fresh lung tumors

A short procedure, recently described by us [34], was employed. Blood clots, necrotic areas and connective tissue capsule were removed and the tumor tissue was rinsed several times in Ca^{2+} and Mg^{2+} containing Dulbecco's phosphate-buffered saline (PBS), pH 7.2. Tumor tissue was cut into 2- to 3-mm fragments and suspended in 15–30 ml of prewarmed enzyme solution (in PBS) consisting of 2 mg/ml trypsin, 2 mg/ml collagenase and 0.2 mg/ml DNase. Mixtures were incubated at 37°C for 5–10 min with shaking and the tumor pieces were then mechanically dispersed, using a Cellector tissue sieve (Bellco Glass, Inc., Vineland, NJ) fitted with a 50-mesh sieve. The dispersed cells were passed several times through a syringe and needle and washed twice at 4°C with RPMI 1640 containing 10% human AB serum or FBS. Cells were suspended in the same medium + 0.2 mg/ml DNase and centrifuged over a 2-layer discontinuous Percoll gradient. The Percoll solution (Pharmacia Fine Chemicals, Uppsala, Sweden) was prepared by mixing 9 parts of Percoll with 1 part of 10× PBS and further diluted to 10–12% (top layer) and 20–22% (bottom layer) v:v with RPMI 1640 containing 10% AB serum. The 2-layer gradient (4 ml each) was prepared in 15-ml conical polystyrene tubes and the cells ($2-4 \times 10^7$ in 2 ml) were overlayed onto the gradient and centrifuged for 7–10 min at 25–50 ***g*** (at the cell–Percoll interface). Large tumor cells (with ⩾80% viability and purity) with some residual macrophages were pelleted to the bottom (the latter could be depleted by a 45- to 60-min incubation at 37°C in plastic dishes), the cells were washed twice with serum-containing medium and then were either used immediately or cryopreserved in DMSO and stored in liquid nitrogen [35].

Human TCGF

Human TCGF-containing medium was obtained from Associated Biomedics System, Inc., Buffalo, NY. It was prepared by stimulating pooled human PBL (1×10^6/ml) for 2–3 days with 1% PHA-M. The medium was concentrated 4-fold with polyethylene glycol, dialyzed and filtered [36]. Depletion of PHA from the crude TCGF (C-TCGF) preparations [37] was performed by 3 consecutive absorptions with packed chicken erythrocytes (10:1 v:v, for 1–2 hr each at 4°C, using a roller drum). This preparation was designated lectin-depleted TCGF (LD-TCGF). The proliferative activity of the C-TCGF and the LD-TCGF was tested at various concentrations (0.15–20%) using a 48- to 72-hr [^{3}H]-thymidine assay in flat-bottom 96-well microtiter plates, employing 5×10^4 TCGF-dependent human CLC or 1×10^5 fresh PBL [5, 36]. While both preparations were equally active with the CLC, the LD-TCGF lost 95–98% of its stimulatory capacity for fresh PBL in comparison with C-TCGF.

Propagation of human lymphoid cells in TCGF

Unfractionated PBL, nylon wool column-nonadherent PBL and Percoll-fractionated cells were cultured for 3–6 weeks with filter (0.2 μ)-sterilized 10–15% v/v C-TCGF or LD-TCGF in RPMI 1640 medium supplemented with 15% serum (see below), 3 mM glutamine, 10 mM HEPES buffer, 1% nonessential amino acids (Grand Island Biological Company, Grand Island, NY), 1% sodium pyrurate (Gibco) and antibiotics. Media contained either heat-inactivated pooled human AB serum or FBS, or fresh autologous serum or plasma. In some experiments 2-mercaptoethanol was used at a final concentration of $1-2 \times 10^{-5}$ M. Cultures were initiated with 5×10^5 cells/ml with C-TCGF and 1×10^6/ml with LD-TCGF in 5–10 ml using 50-ml culture flasks (No. 3013, Falcon Plastics, Oxnard, CA). Cultures were incubated upright in a 7% CO_2 incubator at 37°C and then split every 3–5 days to 1×10^5/ml by the addition of TCGF and culture medium. Occasionally cells were spun down before feeding. With some CLC preparations larger culture flasks (250 ml and 750 ml capacity) were employed. Cell viability was determined by trypan blue dye exclusion.

Before assaying the cytotoxic activity of the CLC, cells were washed twice, 'rested' overnight in RPMI 1640 with 15% AB serum and washed again. All washings were done with 5% serum-containing medium.

Clones of CLC

The clone designated BS-1 was originated from a normal blood donor. T cells were isolated by rosetting with sheep erythrocytes and Ficoll-Isopaque separation, stimulated for 48 hr with

0.0125% v/v PHA-M and seeded in PHA-containing agar [38]. Colonies were picked and recloned at 200 cells per 35-mm Petri dish in agar containing human TCGF. One growing clone (BS-1) was maintained for approximately 4 months using C-TCGF without feeder cells. Clones NN-5 and NN-9 were derived from PBL of a normal blood donor. The cells were cloned in 96-well microplates by limiting dilution, using 1 cell per well, in the presence of irradiated (3000 rad) allogeneic PBL (1×10^5 per well). Clones were supplemented every 5–7 days with fresh medium and TCGF and then expanded for an additional 4 months without feeder cells.

Rosette formation with tumor cells

Tumor cells ($1–2 \times 10^5$ in 0.1 ml) were admixed with 1×10^6 (0.1 ml) fresh PBL or CLC in a 10×70-mm plastic tube. Cells were incubated in a 37°C water bath for 10 min and then spun for 5–10 min at 175 ***g***. The pelleted cells were incubated for 30–120 min at 4°C and resuspended gently with a Pasteur pipette, then 400 cells were examined under the microscope. The number of tumor cells that bound 1, 2, 3 or more lymphoid cells was scored. In addition, conjugates were performed as above, using a 1:1 tumor:lymphoid cell ratio. Rosetting with sheep erythrocytes (SRBC) (for detection of T cells bound to tumor cells) was performed in 2 steps. First, rosettes were prepared as above and resuspended after a 30-min incubation at 4°C. In the second step they were mixed with SRBC (at a 40:1 SRBC:lymphocyte ratio) and the above procedure was repeated. For enumeration rosettes were spun in a cytocentrifuge and stained for 7 min with a 1/15 dilution of Giemsa solution.

Generation of cytotoxic T lymphocytes in vitro

The mixed lymphocyte-tumor cell culture (MLTC) technique was employed [35]. Five million responding patients' lymphocytes were cocultured for 6 days in a 37% incubator with mitomycin C-treated stimulating autologous lung tumor cells at a 2.5–10:1 responder: stimulator ratio. Cultures were carried out in 2 ml enriched RPMI 1640 medium (see above) supplemented with 10% AB serum in 24-well culture plates (Costar, Cambridge, MA) or in 2057 Falcon tubes, in the absence or presence of 10% LD-TCGF. Controls included responder cells alone and responder cells cultivated with LD-TCGF, without stimulating cells. After cultivation cells were washed twice before testing.

^{51}Cr-release cytotoxicity assays

Two to three million cells suspended in RPMI 1640 medium containing 10% AB serum or FBS, glutamine, HEPES and antibiotics were labelled with 200 μCi of [^{51}Cr]-sodium chromate (1 mCi/ml, New England Nuclear Corp., Boston, MA). Normal or malignant lymphoid cells (in 0.5 ml) were labelled in a tube for 2–3 hr at 37°C, and adherent tumor cells were usually labelled for 16 hr (in 5 ml medium) in Falcon 3013 flasks incubated upright at 37°C. Labelled adherent target cells were collected after treating the monolayer for 1–2 min at 37°C solution with EDTA–trypsin (Gibco) containing 0.2 mg/ml DNase. The labelled cells were washed twice in 30 ml 10% serum–RPMI 1640, 'rested' for 2–3 hr at 4°C in the same medium and then washed 2 more times. Similar results were observed with cells labelled and washed with medium containing either AB serum or FBS. When cell aggregates were noticed the labelled cells were passed several times through a syringe with a 23/25-gauge needle. Cell viability was rarely less than 85%, in which case cells were first centrifuged (400 ***g*** for 15 min) over a Ficoll-Isopaque layer and washed twice.

Labelled target cells (10^3 or 10^4 in 0.1 ml) were mixed in triplicate with effector lymphocytes (0.1 ml) at an effector:target cell ratio of 10–30:1 in 96-well, U-shaped microtitration plates (Linbro) using 10% AB serum or FBS/glutamine/HEPES/antibiotics/RPMI 1640 medium. The effector cells were PBL freshly obtained from cancer patients and normal donors, CLC derived from those cells and lymphocytes sensitized in MLTC against the solid tumor cells. Each CLC line was tested weekly for 3–5 consecutive weeks. Controls consisted of target cells incubated alone. Plates were centrifuged at 300 ***g*** for 2 min and then incubated for 6 or 18 hr in a 37°C incubator. Supernates were harvested with the use of a Titertek supernatant collection system (Flow, Rockville, MD). Percent specific ^{51}Cr-release was calculated according to the following formula:

$$\left|\frac{a-b}{c-b}\right| \times 100,$$

where a = count/min of experimental groups, b = count/min of control groups and c = the maximum releasable count/min obtained by incubating the target cells throughout the assay in 5% Triton X-100 (giving 80–92% of the total label incorporated by the cells).

In some experiments effector cells (2×10^6 in 1 ml) were pretreated at 37°C with 2×10^3 units of human fibroblast interferon (HEM Research, Rockville, MD), washed once and tested for cytotoxicity.

Competitive inhibition assays were performed

by adding to the effector–target mixtures various numbers of unlabelled tumor cells (5- to 20-fold the number of labelled target cells) and the assay was then performed as above.

Cell surface markers

Several cell surface markers were analyzed on fresh unfractionated and fractionated PBL and on CLC derived from them, as well as on one of the T cell clones (BS-1).

Binding of SRBC, for enumerating T cells, or of ox erythrocytes coated with 1/100–1/200 dilutions of rabbit anti-ox erythrocyte IgG or IgM (both from Cappel Lab., inc., Cochranville, PA), for detecting cells with Fcγ or Fcμ receptors, was performed by the rosette technique as described above, using an erythrocyte:lymphoid cell ratio of 30–50:1.

Winn neutralization assays

CLC were admixed with either G-11 or HT-29 carcinoma cells at a 10:1 or 15:1 ratio and injected subcutaneously into BALB/c nude mice. Control mice were injected with tumor cells alone. Each inoculum contained 5×10^5 viable tumor cells and each group comprised 5 mice. Tumor diameter was measured with a caliper twice a week for up to 70 days after inoculation.

RESULTS

Expansion of PBL from cancer patients and normal donors with TCGF

A possible clinical application of TCGF in cancer immunotherapy would be to enlarge the number of the patient's own lymphoid cells after antigen-specific or nonspecific stimulation and to infuse large numbers of the autologous, TCGF-expanded cells into the patient. It was therefore of interest to assess the ability of PBL from cancer patients to proliferate in TCGF. Unfractionated PBL from normal donors and patients were cultured with either C-TCGF or LD-TCGF in medium containing either FBS, AB serum, autologous serum or autologous plasma.

Representative cultures of several experiments are shown in Table 1. About 80% of cultures originated with normal PBL expanded 200- to 2500-fold (mean for all normal donors, 638 ± 220), whereas only 37% of the cultures from patients' PBL showed similar growth capacity (mean for all patients, 332 ± 84-fold increase), using C-TCGF and AB serum over a period of 21 days. Five out of thirty-eight (13%) of the cultures from normal donors and 7/27 (26%) of the cultures from patients (5 with lung carcinoma and 2 with breast carcinoma) failed to expand >20-fold. It is still not known whether this difference in growth capacity relates to the age differences between the two groups (see Materials and Methods) or if it is associated with the neoplastic disease. There was no direct correlation between the growth capacity of the individual cultures and their cytotoxic activity (see below). The growth rates in cultures supplemented with FBS and those supplemented with pooled AB serum were similar (data not shown). However, with both normals and patients a greater increase in cell number was observed in cultures fed with medium containing autologous serum (Table 1) or autologous plasma (data not shown) than in AB serum-containing cultures. Addition of 2-mercaptoethanol (optimal concentration, $1–2 \times 10^{-5}$ M) slightly improved the growth capacity of most of the cultures tested (data not shown). The majority of the cultures died when they reached 5–6 weeks, regardless of the type of medium used.

Table 1. Expansion of PBL from normal donors and cancer patients with C-TCGF and LD-TCGF in cultures containing AB serum or autologous serum

Donors	Magnitude of increase in cell number (at 21 days) Autologous serum C-TCGF	AB serum C-TCGF	AB serum LD-TCGF
Patients			
1	315	189	62
2	322	304	112
3	540	129	37
4	1636	1528	402
5	283	432	254
6	98	46	35
7	45	36	17
8	403	397	98
9	244	266	286
110	566	402	244
Normals			
1	1478	570	276
2	1044	651	243
3	202	285	96
4	2672	1480	585
5	818	422	177
6	204	160	82
7	941	254	306
8	166	285	126

The rate of growth in cultures containing LD-TCGF was considerably lower, especially in the first week, than in cultures supplemented with C-TCGF (Table 1). In fact, apparent cell proliferation in LD-TCGF-containing cultures was observed only after 5–8 days of culture.

Unfractionated PBL and nylon wool column-enriched T cells showed similar growth kinetics in C-TCGF and AB serum, with a doubling time of about 30 hr. In contrast, Percoll-enriched LGL had a lower growth rate (doubling time 50–60 hr) than had Percoll-enriched T cells (doubling time 30–40 hr, data not shown).

Binding of CLC to tumor cells

CLC from both patients and normals exhibited high binding capacity to various adherent tumor cells. When centrifuged together at a 5:1 or 10:1 CLC:tumor cell ratio about 50% of the tumor cells bound 3 or more CLC per cell, whereas only 5% of tumor cells bound the same number of fresh PBL (Table 2). When single-cell conjugates (at a 1:1 ratio) were formed 3–10% of fresh PBL and 15–38% of CLC became attached to the tumor cells. No significant differences were observed in binding capacity between normal and patient CLC.

By forming double rosettes using both CLC and SRBC, about 70% of the lymphoid cells attaching to the tumor cells also bound SRBC (Fig. 1).

Cytotoxic activity of CLC derived from unseparated PBL

PBL from normal donors and cancer patients were maintained in culture with either C-TCGF or LD-TCGF in AB serum, autologous serum or FBS. Cytotoxic activity of all those cultures was tested in 6- and 18-hr ^{51}Cr-release assays against various tumor and normal target cells. When cultured cells were tested repeatedly maximum reactivity was reached by 1–2 weeks after culture initiation, and thereafter the pattern and magnitude of reactivity against various targets were quite stable. Similar levels of cytotoxicity were seen in assays performed in the presence of FBS or AB serum (data not shown).

The cytotoxic activity varied among the individual cultures and against the various targets (Fig. 2). Most sensitive to killing were K562 and the adherent tumor lines SK-MES1, MCF7 and G-11, while the least sensitive culture line was HT-29. Fresh lung tumors of autologous or allogeneic origin were also susceptible to lysis by most of the CLC but they were less sensitive than the adherent tumor lines. Moreover, in the 18-hr assay fresh and Con A-stimulated PBL, autologous and allogeneic, were considerably susceptible to lysis. As shown in Fig. 2, the distribution of cytotoxicity of the different effector–target cell combinations was similar (except against HT-29) for CLC derived from patients and normal donors. In general, CLC giving high or low cytotoxicity toward any of the adherent tumor target cells also showed the same pattern against most or all of the other targets, including the fresh tumor and normal cells.

Fresh PBL of both patients and normals also had considerable reactivity (apparently NK activity) against the various adherent tumor cells (especially line 9812) and against K562 but had no significant activity against fresh lung tumor cells or normal lymphoid targets (Table 3). However, following 7 days of incubation in the presence of AB serum (but not in FBS, data not shown) in the absence of TCGF, the spontaneous cytotoxicity was reduced markedly (Table 3). In contrast, TCGF-expanded cells, even in a 6-hr assay, exhibited strong cytotoxicity against K562 and most of the adherent tumor targets, and low reactivity against fresh autologous and allogeneic lung tumors. With the short-term assay no significant reactivity was seen against normal human targets or mouse tumor cells. Prolonging the assay to 18 hr revealed higher reactivity of the CLC against the fresh human tumor cells and also reactivity against fresh or cultured PBL (Table 3).

The data summarized in Table 3 again show that CLC from patients and from normal donors had similar levels of cytotoxicity. No significant differences were found in the extent of lysis of autologous vs allogeneic fresh lung tumor cells and of autologous vs allogeneic fresh PBL and lymphoblasts.

In another series of experiments we tested whether the culture conditions affected the cytotoxic activity. The effect of the serum present in the medium was tested. TCGF-propagated cells cultured in medium containing AB serum or FBS expressed similar patterns of cytotoxicity (data not shown). As shown in Fig. 3, with the majority of the CLC tested the cytotoxic activity by cells maintained with AB serum was higher than that of CLC grown in autologous serum against most of the targets tested. With CLC from

Table 2. Binding of fresh PBL and CLC to tumor cell lines (rosette formation)

Lymphoid cells	Mean % tumor cells that bound 1–2 or ⩾ 3 lymphoid cells*																	
	SK-MES1			9812			CaLu			HT-29			MCF7			K562		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
Fresh PBL ($n=7$)	83	14	5	71	20	9	69	26	5	78	20	4	75	20	5	76	19	5
CLC ($n=16$)	22	26	52	30	23	46	15	27	59	14	23	64	29	22	49	17	39	41

*Percent tumor cells with a = no cells attached, b = 1 or 2 cells attached, c = 3 or more cells attached.

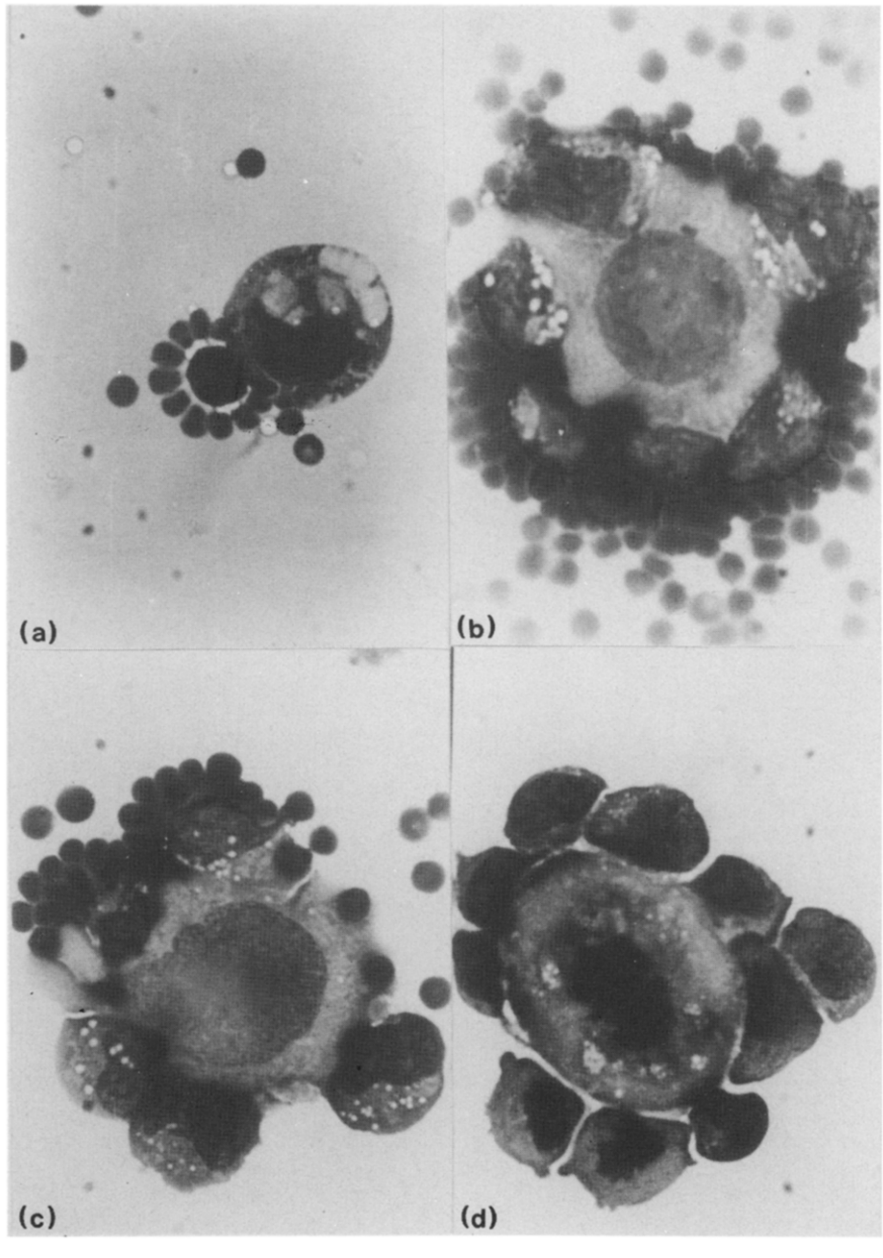

Fig. 1. Rosette formation with sheep erythrocytes of effector cells bound to target cell. (a) Fresh PBL + SRBC attached to MCF7 cell; (b) CLC + SRBC attached to MCF7 cells; (c) CLC + SRBC attached to G-11 cell; (d) CLC without SRBC attached to SK-MES1 cell.

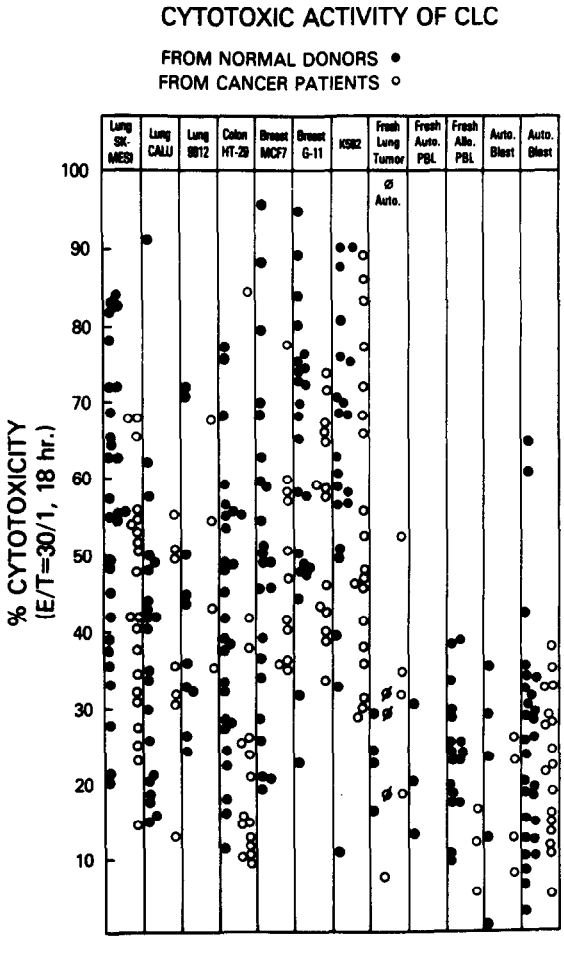

Fig. 2. Cytotoxic activity of individual CLC. Results shown are means of 2–4 separate experiments performed with each culture. Cultures were maintained with C-TCGF and AB serum.

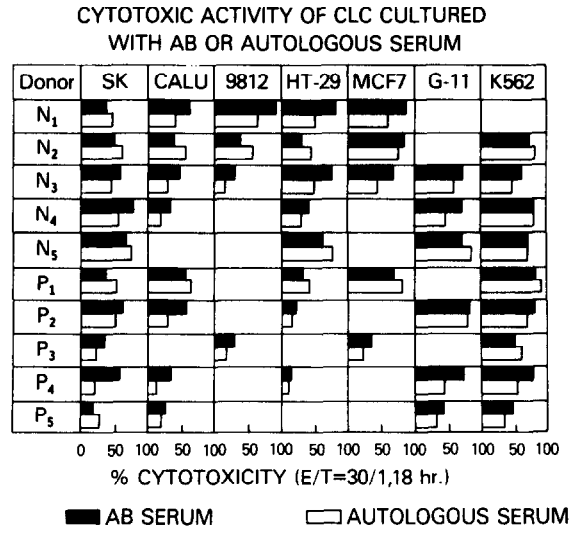

Fig. 3. CLC were grown for 2–5 weeks in medium containing C-TCGF and either AB or autologous serum. The results shown are means of 2–4 separate experiments with each culture.

only a few donors (e.g. N_2 and N_5) cytotoxicity was higher in medium with autologous serum. This was in contrast with the growth pattern, where autologous serum was usually superior to AB serum in promoting CLC growth (Table 1).

A similar inverse relationship between growth rate and cytotoxic activity was found when CLC were maintained with C-TCGF or LD-TCGF. Despite the lower growth rate of CLC in LD-TCGF, most of the CLC tested demonstrated a stronger cytotoxic activity towards most of the

Table 3. Summary of ^{51}Cr-release assays with PBL and CLC from cancer patients (P) and normal donors (N)

	Mean % cytotoxicity (E:T = 20:1) with*									
	fresh PBL		7 days cultured PBL (w/o TCGF)				14–36 days cultured CLC			
	18 hr		6 hr		18 hr		6 hr		18 hr	
	N	P	N	P	N	P	N	P	N	P
SK-MES1	23(12)	19(11)	2(1)	2(1)	11(4)	9(5)	35(6)	29(10)	62(17)	45(15)
CaLu	10(7)	12(6)	4(2)	3(2)	6(3)	4(2)	13(7)	23(9)	37(19)	39(14)
9812†	61(22)	47(16)	4(1)	5(3)	9(5)	12(4)	20(6)	28(7)	44(15)	62(12)
HT-29	21(8)	18(8)	1(1)	2(1)	4(3)	6(2)	18(3)	16(5)	43(17)	34(19)
MCF7	29(17)	30(9)	7(4)	2(1)	10(4)	5(3)	20(3)	25(8)	52(20)	50(13)
G-11†	40(21)	33(16)	6(1)	4(3)	8(1)	11(7)	28(15)	43(5)	63(18)	66(13)
K562†	59(8)	55(10)	3(1)	3(2)	8(3)	5(4)	47(17)	45(17)	68(19)	61(19)
Fresh autologous lung tumor	—	7(4)	—	2(1)	—	5(3)	—	14(4)	—	32(7)
Fresh allogeneic lung tumor	3(2)	6(4)	1(1)	2(1)	4(2)	5(2)	12(2)	15(4)	22(4)	28(13)
Fresh autologous PBL	1(2)	2(2)	—	—	—	—	3(2)	—	14(7)	—
Fresh allogeneic PBL	2(2)	3(1)	4(3)	—	6(2)	—	5(2)	3(2)	17(8)	19(6)
Autologous blasts	2(1)	2(1)	2(2)	—	5(2)	—	5(1)	—	20(11)	—
Allogeneic blasts	1(1)	0(1)	3(2)	—	3(2)	—	7(2)	6(3)	25(13)	20(8)
Mouse M109	—	—	—	—	—	—	—	—	0(1)	1(1)
Mouse YAC-1	—	—	—	—	—	—	—	—	4(2)	9(4)
Mouse RL♂1	—	—	—	—	—	—	—	—	6(2)	15(7)

*Values indicate means (and S.D.). Groups consisted of 4–28 donors with each donor tested 2–4 times. All cultures were medium supplemented with AB serum, and CLC were grown in C-TCGF.

†Target cells highly sensitive to fresh NK cells.

targets (Table 4). Of particular interest was the finding that 3 out of 5 patient CLC exhibited markedly augmented cytotoxicity against the fresh autologous lung tumor.

The cytotoxic activity of CLC from normal donors or patients could be potentiated by pretreating them with human fibroblast interferon (Table 5). A marked increase in cytotoxicity was observed against most of the tumor targets, including fresh autologous and allogeneic lung tumor cells. In contrast, treatment of CLC with interferon resulted in unchanged or lower levels (in 3 out of 5 CLC tested) of cytotoxicity against Con A-induced lymphoblasts (Table 5).

Cytotoxic activity of clonal populations and of CLC derived from PBL enriched or depleted of NK cells

Because of the broad range of target cell killing seen with the CLC, we were interested in analyzing more selected cell populations. In the first series of experiments we tested 3 lymphoid cell clones that had been propagated with C-TCGF for 4 months. The results, shown in Table 6, indicated that 2 of the clones (NN-5 and 9) were highly cytotoxic against SK-MES1 and G-11 targets but had low reactivity against HT-29 and K562. In contrast, clone BS-1 exhibited very strong cytotoxicity against all targets tested. Thus

Table 4. Cytotoxic activity of CLC cultured with C-TCGF (C) or LD-TCGF (LD)

	Mean % cytotoxicity (E/T = 20/1, 18 hr) against*																	
	SK-MES1		CaLu		HT-29		MCF7		C-11		K562		fresh autologous lung tumor		autologous blasts		allogeneic blasts	
Donors	C	LD	C	LD	C	LD	C	LD	C	LD	C	LD	C	LD	C	LD	C	LD
Normals																		
23	21	54	10	21	9	27	—	—	—	—	44	85	—	—	13	16	19	33
24	—	—	23	20	20	22	—	—	44	50	65	67	—	—	24	33	34	39
25	—	—	42	65	46	44	—	—	64	83	81	85	—	—	23	30	30	37
26	—	—	26	30	—	—	—	—	57	61	57	57	—	—	—	—	33	33
Patients																		
30	19	77	—	—	3	9	21	60	8	38	18	76	6	32	—	—	—	—
31	62	60	—	—	—	—	64	64	—	—	75	62	49	31	—	—	—	—
33	28	54	—	—	—	—	20	55	—	—	30	80	18	43	11	19	14	22
34	—	—	—	—	—	—	44	55	43	53	64	64	35	18	—	—	—	—
35	53	45	—	—	12	20	41	35	45	64	37	63	18	32	—	—	—	—
42	31	67	—	—	—	—	—	—	32	83	38	94	—	—	—	—	—	—
43	48	56	—	—	21	41	11	41	58	70	82	83	—	—	—	—	—	—
45	23	64	—	—	15	16	73	99	—	—	55	91	—	—	—	—	—	—
46	32	48	—	—	14	41	65	82	—	—	41	80	—	—	—	—	—	—

*Means of 3 separate experiments.

*Table 5. Effect of interferon on the cytotoxic activity of CLC**

	Mean % cytotoxicity (E/T = 30/1 6 hr) against													
	SK-MES1		HT-29		G-11		MCF7		K562		fresh autologous lung tumor§		blast	
Donor	-†	+‡	-	+	-	+	-	+	-	+	-	+	-	+
Patients														
11	7	35	—	—	—	—	5	12	16	28	7	15	8	2
12	58	62	—	—	—	—	65	56	70	81	38	46	13	4
23	13	35	3	11	22	33	14	17	30	52	7	19	6	5
24	27	49	12	23	48	54	33	48	44	68	—	—	—	—
Normals														
7	8	29	3	10	14	27	15	18	10	19	—	—	—	—
12	9	32	4	11	15	35	11	22	18	28	5	8	—	—
23	29	63	—	—	—	—	21	42	59	72	14	29	8	8
24	25	28	17	24	57	79	49	52	28	45	10	15	14	13

*CLC cultured in C-TCGF with AB serum.
†2×10^6 cells incubated with medium for 2 hr at 37°C.
‡Cells treated for 2 hr at 37°C with 1000–2000 units of human fibroblast interferon and washed once.
§All patient CLC were tested against the autologous fresh lung tumor.

Table 6. Cytotoxic activity of human CLC clones

Clone	% cytotoxicity (E/T = 30/1, 18 hr) Fresh lung tumor	SK-MES1	HT-29	MCF7	G-11	K562
BS-1*	20	93	82	64	80	79
NN-5	—	55	6	—	62	9
NN-9	—	48	16	—	85	26

*Surface marker analysis of clone 1 showed 97% SRBC rosettes and no rosettes with IgG- or IgM-coated ox erythrocytes.

cloned CLC showed some heterogeneity in their patterns of cytotoxicity, but even a clone of effector cells could react against a variety of tumor target cells.

In the next series of experiments we obtained CLC from PBL populations enriched for or depleted of NK cells (as determined by morphology and surface markers) [31] using Percoll gradient centrifugation (Table 7). When tested fresh on day 0 the NK-LGL population possessed higher reactivity than the unseparated cells towards all the targets, whereas the NK-depleted T cell fraction expressed only low cytotoxicity against either NK-sensitive (K562 and G-11) or NK-resistant target cells. After culturing for 2–4 weeks with C-TCGF, the LGL fraction displayed a similar pattern of strong reactivity against all of the cultured tumor targets, substantial reactivity against fresh lung tumor cells and low but significant activity against the allogeneic blasts. In contrast, the cultured T cell fraction showed a remarkably heightened cytotoxic activity (compared with their reactivity on day 0) against all the adherent solid tumor lines as well as against fresh allogeneic lung tumor cells, and also reacted against allogeneic blasts, yet only low levels of cytotoxicity were detected against the NK-sensitive leukemic cell line K562.

In other experiments we found that interferon could boost the cytotoxicity of both types of CLC almost to the same extent against the tumor target cells (data not shown).

An interesting observation was that whereas all the cell populations tested maintained their ability to rosette with SRBC after culturing with TCGF, CLC from PBL or LGL showed a dramatic loss in ability to form rosettes with IgG antibody-coated ox erythrocytes. No rosettes were detected in the CLC populations with IgM-ox RBC (data not shown).

Cold target inhibition experiments

Since the CLC exhibited such a broad range of cytotoxic reactivity, it was of interest to determine whether common or multiple target cell structures were being recognized. Therefore cold target inhibition experiments were performed to determine if various target cells could inhibit lysis of a particular radiolabelled target. The data indicated that K562 could very effectively block cytotoxicity against itself and also against G-11 and SK-MES1 target cells (Table 8). A lesser degree of inhibition was observed against MCF7 and fresh allogeneic lung tumor targets. The murine tumor cell M109, which was resistant to lysis by human CLC (see Table 3), also inhibited the cytotoxicity against all targets poorly. These data suggest that K562 shares a common recognition structure with the other targets, but that some targets (MCF7 and fresh lung tumor) may have other structures not shared with K562.

The recognition by CLC of common target structure(s) is substantiated by the results obtained with the cytotoxic clones (Table 6). To examine this issue further, CLC which reacted with one target were isolated and then tested for reactivity against other targets. This was done by forming CLC–tumor rosettes and separating the rosetted CLC from the unbound CLC using the 2-layer, low-density Percoll technique. Using this procedure we were able to enrich 2- to 3-fold the preparation of CLC that bound to a given tumor target. These enriched cells, however, had levels of cytotoxicity that were comparable to those of unselected CLC, against both the target used for rosetting and with the other target cells. This further suggested that effector CLC may bind via similar surface membrane moieties on various target cells.

Effects of TCGF on mixed lymphocyte–tumor interaction

The experiments described above indicated that TCGF is useful for the nonspecific activation and numerical expansion of cytotoxic cells reactive against tumor cells. It was also of interest to examine whether TCGF could potentiate the generation *in vitro* of specific cytotoxic T cells in a mixed lymphocyte–tumor cell culture (MLTC), as shown recently in a murine tumor system [9, 15].

For this purpose MLTC were performed with

Table 7. Cytotoxic activity and surface markers of Percoll gradient fractionated PBL and of CLC derived from these fractions

Cell fraction	Effectors†	% cytotoxicity (E/T = 30/1, 18 hr) against*							Range of rosette-forming cells	
		K562	SK-MES1	CaLu	HT-29	MCF7	G-11	fresh lung tumor	allogeneic blast	SRBC rosettes	OxRBC-IgG rosettes
PBL nonadherent to nylon wool column	Fresh	59(10)	33(9)	—	22(8)	38(6)	39(5)	9(3)	2(2)	52–84	16
	CLC	67(12)	58(15)	33(9)	46(15)	57(9)	59(17)	22(4)	12(3)	75–94	0–4
Percoll LGL fraction	Fresh	78(11)	55(12)	—	47(9)	47(9)	43(8)	14(4)	—	47–70	20–55
	CLC	74(16)	64(14)	53(17)	65(18)	66(14)	72(19)	32(7)	8(4)	65–83	0–2
Percoll T cell fraction	Fresh	4(2)	8(3)	—	7(4)	6(4)	5(2)	6(2)	—	86–89	1–6
	CLC	15(6)	53(16)	46(12)	44(9)	58(15)	59(21)	23(6)	15(3)	80–92	0

*The data show means (± S.D.) of cells from 7 normal donors.
†Cells were tested fresh and again after 2–4 weeks of culture in medium containing C-TCGF and AB serum.

patients' PBL and autologous fresh lung tumor cells in the presence or absence of LD-TCGF. Cytoxicity against the autologous tumor cells as well as against allogeneic tumor target cells was measured on day 6 or 7. The results shown in Table 9 indicate that TCGF significantly enhanced the cytotoxic response against the sensitizing fresh autologous tumor but at the same time also augmented the non-specific killing against all other targets tested. As expected, PBL cultivated with TCGF without addition of tumor cells also acquired cytotoxic activity, but this was considerably lower against most of the targets than was the reactivity of the MLTC + TCGF group.

Inhibition of tumor growth in nude mice

A local (s.c.) Winn neutralization assay was employed to assess the ability of 3 CLC lines to retard the growth of tumor cells in athymic nude mice. As shown in Fig. 4, admixing of CLC and G-11 tumor cells at a 10:1 or 15:1 ratio completely inhibited tumor growth and all mice survived, in contrast to the death with tumor by day 65 of 8 out of 10 control mice inoculated with tumor alone. With the HT-29 cell line, which showed less susceptibility to lysis *in vitro* than G-11 (see Fig. 2), CLC delayed tumor appearance in all mice, and 2 out of 5 mice did not develop tumors by day 70; in contrast, all control mice had tumors by day 15. These findings indicate that tumor cell killing by CLC can also take place *in vivo*.

DISCUSSION

The results of the present study indicate that the expansion of PBL from patients with advanced carcinoma to large numbers by culture in the presence of TCGF is feasible and that this procedure may be a potentially useful approach to immunotherapy. It was particularly encouraging that the cultured lymphoid cells (CLC) were capable of lysing *in vitro* the autologous fresh tumor cells as well as other target cells. On the other hand, it is worth noting that a larger proportion (>50%) of the 27 patient PBL cultures tested demonstrated a relatively poor growth in TCGF compared to normal PBL cultures. Although such a difference between the normal and the patient groups may relate to the age difference (mean of 32 vs 55 yr), with a lower TCGF responsiveness of PBL from elderly individuals, it is also possible that patient PBL have an inherent deficiency in their responsiveness to TCGF, as has been documented frequently regarding lymphoproliferative responses to PHA or allogeneic leukocytes [39].

In spite of the lower growth capacity of the patients' PBL, no significant differences in

*Table 8. Cold target inhibition of CLC cytoxicity by unlabeled K562 and M109 cells**

Cells	No.	% Inhibition against K562	SK-MES1	MCF7	G-11	lung tumor
K562	1×10^4	51	43	28	43	26
Mouse M109	1×10^4	4	1	11	13	2
K562	4×10^4	85	67	55	85	54
Mouse M109	4×10^4	12	25	29	32	18

*Assay: 2×10^3 ^{51}Cr targets, E:T = 30:1, 6 hr. Values indicate means of % inhibition of 8 donors, relative to controls not containing inhibitor cells.

*Table 9. Effect of LD-TCGF on the induction of cytotoxic activity in autologous mixed lymphocyte-tumor culture**

Patient	Culture	% Cytotoxicity (E:T = 20:1, 16 hr) against SK-MES1	HT-29	MCF7	G-11	K562	autologous lung tumor (fresh-frozen)
22	PBL alone	3	1	2	5	2	1
	PBL + tumour	4	2	5	8	6	4
	PBL + TCGF	8	5	5	19	11	6
	PBL + TCGF + tumour	9	14	16	23	29	20
26	PBL alone	3	4	4	4	3	2
	PBL + tumour	5	6	9	10	7	7
	PBL + TCGF	9	7	10	13	17	13
	PBL + TCGF + tumour	16	14	24	23	21	28

*Cytotoxicity was measured on days 6 (donor 1) and 7 (donor 2). Stimulation was done at a responder/stimulator ratio of 5:1 (No. 1) and 10:1 (No. 2). LD-TCGF was added on day 0 at a final concentration of 10% v/v.

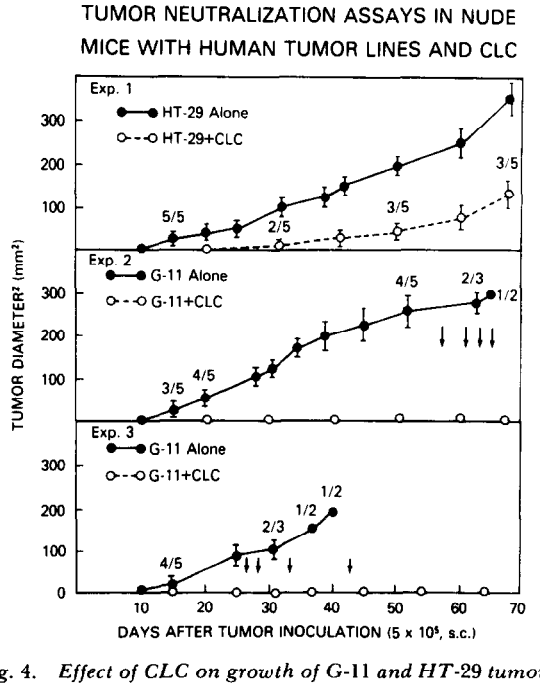

Fig. 4. Effect of CLC on growth of G-11 and HT-29 tumors in BALB/c nude mice. Figures show No. of mice with visible tumor/total No. of surviving mice at various time points. Arrows indicate death of mice with progressive tumors. Groups consisted of 5 mice each. In experiment 1 CLC from a normal donor N_{14} were injected with the tumor cells at a ratio of 10:1, in experiment 2 CLC from donor N_{22} were injected at a 15:1 ratio and in experiment 3 CLC from donor N_{35} were injected at a 10:1 ratio.

cytotoxic activity against a variety of tumor target cells were noticed between the two groups. The ability of TCGF-expanded human CLC to lyse fresh autologous and allogeneic solid tumor cells *in vitro* has also been reported recently by two other groups [21–23]. The additional information in the present study is that such cultured cells are also highly cytotoxic to K562 and a variety of adherent tumor cells, both having low and high susceptibility to lysis by fresh NK cells. Furthermore, in agreement with one of the above-cited studies [21] but in contrast with others [7, 8, 23], we have also found significant cytotoxic reactivity against fresh and cultured autologous as well as allogeneic PBL. It should be pointed out, however, that the antinormal but not the antitumor reactivity was only demonstrated in our study when the ^{51}Cr-release assay was extended from the conventional 4- to 6-hr assay to a 16- to 18-hr assay.

The nature of the effector cells in the human CLC that are responsible for the cytotoxicity against the broad range of target cells has not been fully defined. However, recent studies in our laboratory [7, 8, 18, 24, 26] have indicated at least 4 types of cytotoxic activities by CLC: NK-like activity, antibody-dependent cell-mediated cytotoxicity (ADCC), lectin-dependent cell-mediated

cytotoxicity and polyclonal activation of T cells. Much of the cytotoxicity seen in the present study is probably attributable to propagation of NK cells. Most of the targets that were highly susceptible to lysis by CLC were also susceptible to lysis by fresh NK cells. Further, as a characteristic feature of cytotoxicity of NK cells but not of human cytotoxic immune T cells [40], interferon pretreatment of the CLC caused a substantial increase in reactivity against most target cells. In addition, culturing of LGL, which account for the NK and ADCC activities of human PBL [31], resulted in CLC with the same characteristics as those of cultured PBL, whereas cultures of purified small T cells showed a different, more restricted pattern of cytotoxic activity.

These observations suggest that TCGF can support the proliferation of NK cells and maintain or, as found recently [41, 42], augment their cytotoxic activity. In addition, it may promote the polyclonal activation and/or differentiation of precursors of cytotoxic T effector cells. This is suggested by the observation that the Percoll fraction enriched for T cells and depleted of LGL and NK activity, which had no detectable cytotoxicity before culture against any of the targets, upon culturing with TCGF developed substantial levels of cytotoxicity against alloblasts and fresh or cultured adherent tumor cells but little or no reactivity against the NK-sensitive K562. The reactivity against the alloblasts is probably due to polyclonal activation of cytotoxic T cells, as has been previously described for human CLC [7, 8, 18, 24, 25]. Such cells probably also account for the reactivity against allogeneic normal lymphoid cells and blast cells seen with CLC initiated from PBL. It remains to be determined whether the reactivity of the cultured T cells against tumor target cells is also due to polyclonally activated T cells. One piece of evidence against this is that such reactivity could be augmented by interferon. An alternative and more likely explanation is that NK-like cells or other natural effector cells can develop from inactive precursors that are present in the small T cell fraction.

The differences in reactivity patterns by CLC derived from LGL-enriched or LGL-depleted Percoll fractions support the previous suggestion [18, 26] of heterogeneity of effector cells in CLC. The tests with the 3 clones of CLC provide further support for separate subpopulations of effector cells since 2 of the 3 clones had a more restricted pattern of cytotoxicity than the third, reacting strongly against adherent tumor targets but not against K562. From the limited testing done so far, these latter clones appear to fall in the same category as the CLC derived from the LGL-depleted T cell fraction. However, the degree of heterogeneity within the CLC appears to be rather limited. The effector cells appear to recognize target structures that are common to a wide range of cell lines (and which are probably less common on fresh tumor cells), as indicated by the cold target inhibition experiments and the reactivity pattern of positively selected target-binding CLC. To accurately determine the extent of heterogeneity of effector cells it will be necessary to develop techniques to obtain large numbers of clones with high efficiency. Unfortunately, to date it has been quite difficult to isolate and continue to propagate cytotoxic clones of human CLC and our efforts have been severely limited by this technical problem.

In addition to TCGF, there may be other factors in the supernatant of PHA-stimulated cells that can contribute to the changes in the cytotoxicity patterns of cultured cells, such as residual lectin (even in LD-TCGF preparations), interferon and other lymphokines. Crude preparations of TCGF contain relatively large quantities of interferon which is known to boost NK activity [40, 43].

The observation that CLC grown in LD-TCGF had a higher cytotoxic activity compared with CLC grown in C-TCGF suggests that either continuous presence of the lectin suppresses or somehow blocks the cytotoxic cells or that LD-TCGF is more capable of selecting for the small subpopulation of activated NK or polyclonally activated cytotoxic T cells which are already present in fresh blood and which express the TCGF receptor on their membranes.

The ability to generate lymphocytes with antitumor autoreactivity with TCGF may be sufficient to consider re-infusing them in the autologous patient after the bulk of the tumor mass has been reduced by other means (i.e. surgery, chemotherapy, radiotherapy). However, a more powerful antitumor reactivity may be attained by first stimulating in culture the patient's PBL with autologous or pooled allogeneic tumor cells and subsequently expanding the activated cells to large numbers with TCGF. Generation of clonal populations with specific antitumor reactivity and devoid of antinormal reactivity may even further increase the immunotherapeutic efficacy of such cells, although tremendous efforts will have to be made to obtain such cells in sufficient numbers. The present observation that addition of TCGF to autologous MLTC can potentiate the antitumor cytotoxic reactivity of the sensitized cells offers another useful application of TCGF.

As an initial indication of possible *in vivo* antitumor effects, we have shown that human

CLC can inhibit the growth of human tumor cells in nude mice in a local Winn assay. However, other recent studies have already indicated some of the difficulties involved in using TCGF-propagated cells *in vivo*. Thus systemic infusion into tumor-bearing mice has shown no or, at best, limited beneficial effects [16, 44, 45]. As CLC are TCGF-dependent and poorly able to circulate when administered systemically [46], methods for overcoming these limitations need to be developed. Repeated injections of highly concentrated and/or purified preparations of TCGF, repeated injections of large numbers of CLC systemically, intralesionally and/or into blood and lymph vessels leading to tumor area, and temporary blockade of the reticuloendothelial system may be helpful.

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