Eur J Cancer Clin Oncol, Vol. 18, No. 10, pp. 991–1000, 1982.
Printed in Great Britain.

0277-5379/82/100991-10$03.00/0
Pergamon Press Ltd.

A Rapid Technique for Isolation of Viable Tumor Cells from Solid Tumors: Use of the Tumor Cells for Induction and Measurement of Cell-mediated Cytotoxic Responses

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Abstract—*A rapid and simple technique for the isolation of viable tumor cells from human and mouse solid neoplasms is described. It consists of a 5 to 10-min treatment with trypsin–collagenase–DNase mixture, followed by mechanical disaggregation of the tumor tissue and subsequently by a brief centrifugation on a discontinuous Percoll gradient. With the tumors employed, this procedure usually requires less than* 1 hr *and results in preparations comprising greater than* 80% *tumor cells with viability of* 80–90%. *Cell-mediated cytotoxic response was measured with: (a) unsensitized lymphocytes freshly obtained from tumor-bearing hosts; (b) lymphocytes propagated in culture with T cell growth factor; and (c) lymphocytes stimulated in cocultures with autologous or syngeneic tumor cells. The cytotoxic activity was assessed in a modified* [^{51}Cr]*-release assay adapted for solid tumor cells, allowing a long incubation period* (24 hr) *and the use of a low number* (200–1000) *of highly labeled target cells* (2–10 *counts/min/cell*).

INTRODUCTION

CARCINOMAS and sarcomas constitute about 90% of all human malignancies. Despite this fact, most studies to date dealing with *in vitro* assessment of cell-mediated cytotoxicity in both experimental and human tumors have been with neoplasms of lymphoid origin or with tissue culture lines derived from solid growths. The scarcity of attempts [1] to evaluate cytotoxic responsiveness to tumor cells freshly obtained from solid tumor tissues may be attributed mainly to the difficulties encountered with the isolation of such cells and the lack of convenient assay techniques. Although several procedures are now available for the isolation and purification of solid tumor cells and the infiltrating effector cells [1–3], they are lengthy, tedious and sometimes require sophisticated equipment. Freshly obtained solid tumor cells are frequently unsuitable for the routine radioisotopic assays, due to insufficient incorporation of the label or to high spontaneous isotope release during a long incubation period that is usually required to demonstrate appreciable target cell damage.

In the present study we describe a rapid and simple technique for the isolation of viable tumor cells from solid neoplasms and a modification of the [^{51}Cr]-release assay for the assessment of cell-mediated cytotoxicity to such cells.

Accepted 12 May 1982.

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‡Barbara L. Ikejiri was supported by a research grant from the late Mr. Harold B. Abramson, New Jersey, to the Lautenberg Center.

MATERIALS AND METHODS

Mouse tumors

Madison 109 (M109), a spontaneous lung carcinoma of BALB/c mice [4], was maintained *in vitro* and *in vivo* by biweekly serial passages subcutaneously or intramuscularly. Primary spontaneous mammary tumors [5] were obtained from 8 to 12-month-old C3H/He NMTV+ mice. They were used as such or

after a single passage *in vivo* or *in vitro*. MBL-2, a Moloney virus-induced lymphoma of C57BL/6 mice, was obtained from the ascitic fluid, and YAC-1, a Moloney virus-induced lymphoma of A/Sn mice, was maintained as a tissue culture line. Cultures of tumor cells were carried out in RPMI 1640 medium supplemented with 3 mM glutamine, 10% heat-inactivated fetal bovine serum (FBS) and 50 μg/ml of gentamicin. Primary mammary tumor cultures were established in the above medium fortified with 5×10^{-5} M 2-mercaptoethanol (2-ME), 1% nonessential amino acids (Gibco, Grand Island Biological Company, Grand Island, NY) and 1% sodium pyruvate (Gibco).

Human tumors

Tumor biopsies excised from previously untreated patients with squamous cell (7 patients) and adenocarcinoma (5 patients) of the lung were received 2–8 hr after surgery. They were stored on ice in RPMI 1640 medium with antibiotics until processing. The following tissue culture lines were maintained in FBS–glutamine–antibiotics–RPMI 1640 medium: SK-MES 1, CaLu and 9812, derived from patients with lung carcinomas; HT-29, derived from a colon carcinoma; MCF7 and G-11 from breast carcinomas; and K562 from a patient with chronic myeloid leukemia in blast crisis.

Lymphoid cells

Heparinized patients' blood, drawn before tumor excision, was separated on a Ficoll-Hypaque layer (500 *g*, 30 min) and the mononuclear cells (PBL) were washed twice. Mouse splenocytes were harvested from M109 tumor-bearing animals by teasing apart the spleens with forceps in 2% FBS–RPMI 1640 medium and then passing the cells several times through a syringe. The single cell suspension was centrifuged on a Ficoll-Hypaque layer (400*g*, 12 min) and the mononuclear cells washed twice. Cell viability was determined by trypan blue exclusion. Lymphoblasts were obtained by culturing for 3 days human and mouse lymphocytes (1×10^6/ml) in 10% serum–RPMI 1640 medium containing 0.5% PHA-P (Difco) or 2 μg/ml Con A (Miles-Yeda) respectively. All human and mouse lymphoid cell cultures were carried out in 10% AB serum or FBS respectively.

Isolation of tumor cells from solid tumors

Mouse tumors (1–1.5 cm diameter) and human biopsies (1–3 g) were processed within 1 hr and 2–8 hr after excision respectively. Blood clots, necrotic areas and connective tissue capsules were removed, followed by rinsing the tumor tissue several times in Ca^{2+} and Mg^{2+}-containing Dulbecco's phosphate-buffered saline (PBS). Tumor tissue was cut into 2 to 3-mm pieces and suspended in 15–30 ml of prewarmed enzyme mixture in PBS consisting of 2 mg/ml trypsin (type III, 10,000 U/mg protein, Sigma Chemical Co., St. Louis, MO), 2 mg/ml collagenase (type I-S, 180 U/mg solid, Sigma), 0.2 mg/ml DNase (type I, 1548 U/mg protein, Sigma) and antibiotics. Mixtures were incubated at 37°C for 5–10 min with intermittent shaking or on a magnetic stirrer, then subjected to gentle mechanical dispersal, using a stainless steel Cellector tissue sieve fitted with a 50 mesh sieve and a glass pestle (Bellco Glass Inc., Vineland, NJ). The resultant cell suspension was passed gently several times through a syringe equipped first with a 20-gauge and then with a 23 or 25-gauge needle. If the percentage of cell viability at that point was less than 80%, the cell suspension was further incubated at 37°C for 5 min. To some tumor preparations more DNase was added to the cell suspension to avoid excess gel formation. Cells were then washed twice at 4°C in 10% human AB serum (for human cells) or FBS (for mouse cells) in RPMI 1640 medium. Cells were suspended in 10% serum–RPMI 1640 containing 0.2 mg/ml DNase. In the case of large cell aggregates, a syringe was used (see above) to disperse the cells.

Cell fractionation was performed in either Percoll (polyvinylpyrrolidone-coated colloidal silica, Pharmacia Fine Chemicals, Uppsala, Sweden) or bovine serum albumin (BSA fraction V, Sigma) discontinuous gradients. The Percoll solution was prepared by mixing 9 parts, of Percoll with 1 part of ×10 concentrated PBS, pH 7.2, and then the appropriate dilutions were made in 10% serum–RPMI 1640. BSA was dissolved in PBS to a final concentration of 35% w/v, adjusted with HCl to pH 5.1–5.2 and diluted as above. Under standard conditions (see Results) both types of gradients were made of two layers, 4–5 ml each, in 15 ml conical plastic tubes. BSA was used at concentrations of 17.5–20% (bottom layer) and 10–12.5% (top layer); Percoll was used at 20–22% and 10–12.5% respectively. Tumor cell suspensions ($20–40 \times 10^6$ cells in 2–3 ml) were carefully overlaid on the gradients and centrifuged for 7–10 min at 400–650 rev/min (20–55 *g* at the cell–gradient interface), depending on tumor preparation (see Results), using a Beckman TJ-6 centrifuge. Cells from the various fractions were collected with a pipette, washed once or twice with serum-containing medium and examined for

viability (trypan blue exclusion) and cell composition (cytocentrifuge preparations stained with Giemsa). Whenever tumor cell fractions were contaminated with greater than 10% phagocytic cells (see below), adherent cells were adsorbed onto plastic dishes for 30–45 min at 37°C in 10% serum-containing medium.

Cell fractions enriched in tumor cells were either used immediately, cryopreserved in liquid nitrogen [6] or cultivated in tissue culture flasks with RPMI 1640 medium supplemented with 3 mM glutamine, 10 mM Hepes buffer, 10–15% FBS, 5×10^{-5} M 2-mercaptoethanol (2-ME), 1% nonessential amino acid solution, 1% sodium pyruvate solution, 1% antimycotic–antibiotic solution (all from Gibco) and 25 μg/ml gentamycin (enriched RPMI 1640 medium).

Enumeration of phagocytic cells

Cell suspensions in 10% serum were incubated for 60 min at 37°C with Bacto-Latex particles (0.81 μ, Difco), one drop per ml, with intermittent agitation, washed twice and observed under the microscope. In some experiments Fc receptor-bearing cells were determined by rosetting with erythrocytes (ox for human and sheep for mouse) coated with anti-erythrocyte IgG [7]. Usually the percentage of Fc^+ cells was slightly higher than the percentage of phagocytic cells.

Propagation of T lymphocytes with T cell growth factor (TCGF)

Human PBL and mouse splenocytes derived from tumor-bearing hosts were propagated (cultured T cells, CTC) for 1 month and 3 months respectively with crude TCGF preparations [8] at 10–15% v/v in RPMI 1640 medium supplemented with 10% FBS (for mouse cells) or 15% AB serum (for human cells), 3 mM glutamine, 10 mM Hepes buffer, 1% nonessential amino acids, 1% sodium pyruvate, 2-ME (1×10^{-5} M for human cells and 5×10^{-5} M for mouse cells) and antibiotics. 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 supernate was collected, concentrated ×4 with polyethylene glycol and dialyzed [9]. For cultivating mouse cells, TCGF was prepared from splenocytes of W/Fu rats (5×10^6/ml) stimulated for 40 hr with 5 μg/ml Con A in enriched RPMI 1640 medium, the cells were pelleted (800 **g**, 20 min), and the supernate collected and filtered through a 0.22 μm filter. Cultures were initiated at 5×10^5 cells/ml and subsequently split every 3–5 days to 1×10^5/ml with the addition of fresh crude TCGF and culture medium. Before assaying the cytotoxic activity of the cultured cells, residual mitogenic material was removed as follows: human cells were washed twice, 'rested' overnight in 10% AB serum–RPMI 1640 and washed again; mouse cells were washed once, incubated for 30 min at 37°C with 10 mg/ml of α-methyl-D-mannoside and washed two more times. All washings were done with 5% serum-containing medium.

Induction of cytotoxic cells in vitro

The mixed lymphocyte–tumor cell culture (MLTC) technique was employed to generate cytotoxic lymphocytes [10]. Five million responding lymphocytes were cocultured for 6 days in a 37°C incubator with mitomycin C-treated stimulating tumor cells in 24-well culture plates (Costar, Cambridge, MA) in a total volume of 2 ml enriched RPMI 1640 medium (see above) supplemented with 10% AB serum (for human cultures) or 10% FBS (for mouse cultures). The optimum responder/stimulator cell ratios were 2.5–10/1 for human MLTC, 20–100/1 for freshly obtained and 200–800/1 for cultured murine M109 tumor cells. After cultivation the cells were washed twice and the number of viable cells was determined with trypan blue.

[^{51}Cr]-*labeling of target cells*

The foliowing cell types were labeled: frozen–thawed fresh tumor cells, freshly harvested tumor cells, cultured tumor cells (either derived from lines or from primary cultures) and mitogen-induced lymphoblasts. Adherent tumor cells were detached from the flasks with trypsin–EDTA solution (Gibco) and washed twice with 10% serum–RPMI 1640. Two to three million cells suspended in glutamine–Hepes–antibiotics–RPMI 1640 medium were labeled with 200 μCi of [^{51}Cr]-sodium chromate (1 mCi/ml, New England Nuclear Corp., Boston, MA) using three different techniques. In the first, cells were incubated in a total volume of 1 ml without serum in 50-ml plastic conical tubes for 1–2 hr at 37°C with intermittent shaking and then washed three times with 40 ml cold RPMI 1640 medium without serum. In the second, cells were labeled as above but with the presence of 10% serum (AB or FBS) during incubation with [^{51}Cr] and washing. In the third, cells were suspended in 5 ml medium containing 10% serum and incubated upright in 50 ml culture flasks (No. 3013, Falcon Plastics, Oxnard, CA)

for 16 hr. In the latter procedure, adherent labeled tumor cells were harvested from the flasks, either by a thorough pipetting or by first removing the nonadherent cells and then adding 1 ml of prewarmed trypsin–EDTA solution (Gibco) containing 0.2 mg/ml DNase to both the nonadherent cells and the flask. After 1–2 min at room temperature, the adherent cells were removed from the flask and pooled with the nonadherent cells. The labeled cells were washed twice in 10% serum, 'rested' for 2–3 hr at 4°C or room temperature in 10% serum and then washed two more times with 10% serum in RPMI 1640 medium (similar results were obtained with AB serum and FBS). Where cell aggregates were noticed, the labeled cells were passed several times through a syringe fitted with a 23 or 25-gauge needle before using in the [^{51}Cr]-assay. Rarely cell viability was less than 85%; in that case cells were first centrifuged over a Ficoll-Hypaque layer (400*g*, 15 min) and washed twice before use.

Measurement of cell-mediated cytotoxicity

Labeled target cells (10^3 or 10^4 in 0.1 ml; see Results) were mixed in triplicate with effector lymphocytes (0.1 ml) at an effector/target cell ratio of 20/1 in 96-well U-shaped microtitration plates (Linbro), using 10% FBS–glutamine–Hepes–antibiotics–RPMI 1640 medium. The effector cells were: PBL or splenocytes freshly obtained from cancer patients and M109 tumor-bearing mice, respectively; CTC derived from those cells; or lymphocytes sensitized in MLTC against the solid tumor cells. Controls consisted of target cells incubated alone. Plates were centrifuged at 300*g* for 2 min and then incubated for 4–24 hr in a 37°C incubator. At various times (see Results) supernates were harvested with the use of a Titertek supernatant collection system (Flow, Rockville, MD). Percentage specific [^{51}Cr] release was calculated according to the following formula:

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

where a is the counts/min of experimental groups, b is the counts/min of control groups and c is the maximum releasable counts/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).

RESULTS

Isolation of tumor cells

In preliminary experiments attempts were made to disperse the tumor tissues by either mechanical means or a brief (5–10 min) enzymatic digestion. Whereas the first procedure yielded a larger number of cells, cell viability was only 15–30%, in comparison with 80–90% in the latter method. Increasing the incubation time to 30–60 min with the enzyme mixture, or repeated additions (2 or 3 times) of fresh enzyme solution for intervals of 15–20 min each, resulted in a considerable increase in the number of viable cells. Subsequently we found that the disaggregation procedure can be shortened and improved by first exposing the tumor tissue for 5–10 min to the enzymes followed by mechanical dispersal. Using this technique, $2–7.5 \times 10^7$ viable cells could be isolated from the human carcinomas and $4–12 \times 10^7$ from the mouse tumors (per 1 g tissue), with cell viability of 80–95%.

Analysis of cell composition of the disaggregated tumors (Table 1) revealed that the frequency of tumor cells in the human preparations ranged from 40 to 60%; the remaining cells were identified as lymphocytes, macrophages and normal parenchymal cells in various proportions. A higher proportion of tumor cells was noticed in the mouse tumor preparations, especially in the M109 tumor (60–83%). Cell fractionation employing centrifugation in Percoll and BSA gradients of low density and for a short period of time (7–10 min) proved useful for the enrichment of tumor cells (Table 1). Usually more reproducible results were obtained with the Percoll technique and it was therefore adopted for routine use. Highest yields of tumor cells and minimal contamination with normal elements were attained by using two layers of Percoll, of 12% (top) and 20–22% (bottom). With the M109 tumor and the majority of the human tumors, centrifugation at 400 rev/min (approximately 20*g*) for 7–8 min was sufficient, whereas with the mammary tumor and some of the human tumors a higher centrifugal force was required—up to 650 rev/min (approximately 55*g*) for 10 min.

Tumor cells with the least contamination by normal cells were found primarily in the cell pellet. Frequently cells banding on top of the bottom Percoll or BSA layer were also enriched in tumor cells, and they were pooled with the cells in the pellet fraction. In general, 50–70% of the tumor cells found in the original tumor cell preparation could be recovered in 70–90% purity in those fractions. The tumor cell fractions were depleted of erythrocytes and lymphocytes (which remained in the top layer), but sometimes consisted of 10–20% phagocytic cells.

*Table 1. Cell composition of human and mouse solid tumor preparations, before and after fractionation**

Tumor†		Total number of viable nucleated cells ($\times 10^{-6}$) recovered from 1 g tumor tissue	Percentage viable cells	Percentage tumor cells	Percentage phagocytic cells	Percentage lymphoid cells	Presence of erythrocytes
Human lung carcinoma (6)	Unfractionated	57(12)	83(4)	54(8)	13(6)	17(6)	++
	Fractionated‡	26(8)	89(5)	83(9)	5(3)	2(2)	+−
Mouse lung carcinoma M109 (11)	Unfractionated	79(7)	86(8)	72(9)	10(3)	6(4)	+
	Fractionated‡	44(9)	90(3)	89(4)	4(3)	1(1)	−
Mouse mammary tumor (8)	Unfractionated	68(10)	85(9)	59(10)	21(8)	13(5)	++
	Fractionated‡	37(7)	85(7)	82(5)	11(4)	3(2)	+−

*Values indicate means (and S.D.).
†In parentheses—number of tumors processed.
‡Pellet obtained from Percoll gradients (see Materials and Methods).

Most of the phagocytes, but not the tumor cells, could be depleted from the tumor cell fractions by adsorption for 30–45 min at 37°C onto plastic dishes, thereby resulting in cell preparations composed of 85–95% tumor cells. In several experiments three layers of Percoll (10, 15 and 20%) were used, with most of the lymphocytes localized above the 15% layer and the tumor cells above the 20% layer and in the pellet.

Tumor cells thus obtained were capable of producing confluent adherent monolayers (primary cultures) within 3–6 days after seeding at $0.3–1 \times 10^6$ cells/ml in enriched RPMI 1640 medium. The isolated M109 and the mammary tumor cells also formed visible growths within 2 weeks after inoculating 1×10^5 cells subcutaneously or intramuscularly into syngeneic mice.

[^{51}Cr]-labeling under modified conditions

Tumor cells from solid neoplasms, in contrast to lymphoid tumors, usually require long incubation to demonstrate cell-mediated cytotoxicity. Prelabeling of solid tumor cells with [^{51}Cr] by the standard technique [11] usually results in a high spontaneous release of the label, and therefore assays are limited to short periods of time (4–6 hr). Moreover, freshly harvested solid tumor cells frequently incorporate only small amounts of [^{51}Cr], thereby necessitating relatively large quantities of labeled target cells and, accordingly, large numbers of effector cells for assays. However, both types of cells may be available in only limited amounts in human systems.

As shown in Tables 2 and 3, [^{51}Cr]-labeling for 1–2 hr (standard technique) resulted in target cells that incorporated 400–1200 counts/min/1×10^3 cells, whereas increasing the labeling time to 16–18 hr yielded a 5 to 10-fold increase in cell radioactivity. This held for both fresh and cultured adherent carcinoma cells; nonadherent tumor and normal cells of lymphoid origin also incorporated higher amounts of [^{51}Cr] but the increase was less impressive compared with that seen with the solid tumors. Other advantages of the long labeling are: (a) tumor cells that have been isolated from solid growths can recuperate from the traumatic treatment during tumor disaggregation; and (b) phagocytic cells and parenchymal cells (e.g. fibroblasts) which usually contaminate tumor cell preparations bind strongly to the culture flask, in contrast to the majority of the tumor cells, and are not detached upon harvesting the labeled cells by pipetting or a brief treatment (1–2 min) with trypsin–EDTA.

While the amount of [^{51}Cr] incorporated and the level of spontaneous release were not significantly influenced by the presence or absence of serum during a short period of labeling, the presence of serum was of paramount importance during washings, especially with fresh solid tumor cells and with the adherent cultured lines. Target cells labeled and washed without serum showed poor viability (40–70%) and released 19–58% of the label spontaneously at 4–6 hr of incubation, and up to 75% at 24 hr. The spontaneous release could be reduced to 1/3 when cell washings after labeling were carried out in serum (5–10%)-containing medium. For long-term incubation assays (16–24 hr),

Table 2. [^{51}Cr] *Uptake and spontaneous release using human adherent and nonadherent target cells*

Cells	Radioactivity (counts/min) incorporated by 1×10^3 cells labeled for:		Percentage spontaneous [^{51}Cr] release from cells incubated for:			
	1–2 hr	16–18 hr	4–6 hr		8–10 hr	16–24 hr
Fresh lung carcinoma	380(240–480)*	2470(1100–3900)*	25(19–34)†	12(8–14)‡	15(10–19)‡	20(16–23)‡
SK-MES 1	490(280–720)	4520(2750–9700)	26(20–37)	13(10–17)	17(14–25)	21(18–31)
CaLu	1220(660–1800)	4890(2400–7530)	28(22–35)	14(9–23)	ND§	25(21–30)
9812	720(350–1370)	3550(2050–5060)	ND	ND	17(15–20)	28(19–36)
HT-29	360(310–490)	2840(1330–6300)	ND	8(5–12)	ND	14(9–18)
MCF7	1150(560–1700)	7530(3850–12700)	ND	9(5–11)	16(14–18)	18(13–32)
G-11	1240(780–1660)	5190(2700–7980)	ND	16(8–22)	19(12–24)	22(17–33)
K562	560(270–960)	4750(2900–9880)	20(14–26)	12(9–18)	ND	15(11–21)
Lymphoblasts (PHA)	370(220–470)	710(540–990)	ND	16(14–20)	ND	34(22–45)

*Target cells ($2–3 \times 10^6$) labeled with 200 μCi $Na^{51}CrO_4$. Values represent means (and range) of 5–18 separate experiments.

†Target cells labeled without serum, washed 3 times without serum and used immediately. Values show means (and range) of 3–6 separate experiments.

‡Target cells labeled in the presence of 10% FBS or human AB serum, washed twice, 'rested' for 2–3 hr and again washed in serum-containing medium. Values show means (and range) of 5–20 separate experiments.

§Not done.

Table 3. [^{51}Cr] *Uptake and spontaneous release using mouse adherent and nonadherent target cells*

Cells	Radioactivity (counts/min) incorporated by 1×10^3 cells labeled for:		Percentage spontaneous ^{51}Cr release from cells incubated for:			
	1–2 hr	16–18 hr	4–6 hr		8–10 hr	16–24 hr
Fresh M109	1250(660–1700)*	4870(2600–6140)*	46(36–58)†	14(11–17)‡	16(13–21)‡	25(15–30)‡
Cultured M109	870(650–1820)	3720(2300–7240)	39(25–48)	13(9–19)	15(12–16)	21(14–24)
Fresh mammary tumor	910(470–1240)	3200(2830–6250)	ND§	ND	19(12–26)	29(21–33)
Cultured mammary tumor	780(610–1400)	3160(2240–5580)	27(19–36)	10(7–13)	ND	24(14–27)
Fresh MBL-2	640(380–1100)	2200(1300–2800)	ND	ND	ND	27(25–33)
Cultured YAC-1	490(280–890)	1980(1360–3570)	ND	13(8–15)	16(10–24)	24(19–35)
Lymphoblasts (Con A)	480(270–690)	1470(1120–1960)	21(16–27)	13(9–16)	ND	27(22–39)

*Target cells ($2–3 \times 10^6$) labeled with 200 μCi $Na^{51}CrO_4$. Values represent means (and range) of 4–15 separate experiments.

†Target cells labeled without serum, washed 3 times without serum and used immediately. Values show means (and range) of 3–7 separate experiments.

‡Target cells labeled in the presence of 10% FBS, washed twice, 'rested' for 2–3 hr and again washed in FBS-containing medium. Values show means (and range) of 5–20 separate experiments.

§Not done.

'resting' of the target cells for 2–3 hr followed by additional washings immediately before using in the assay was found very useful and spontaneous release with most tumor cell preparations was in the range of 15–25% (Tables 2 and 3).

Freshly harvested solid tumor cells could also be maintained viable and functional for 1–3 days without adhering to the culture flasks by using serumless medium (Gibco), thus avoiding the need for trypsinizing the cells before or after labeling. However, washing and 'resting' required serum or 1% BSA to lower the spontaneous release (data not shown).

Cytotoxic activity against solid tumor cells

In another series of experiments we compared freshly harvested carcinoma cells and cultured tumor cells (either derived from continuous lines or primary cultures) for their ability (a) to induce *in vitro* syngeneic (mouse) and autologous (human) cytotoxic effector cells, and (b) to serve as target cells in the [^{51}Cr] release assay. In addition, we tested whether the labeling conditions (1 hr vs 16 hr plus 'resting') affected the ability of the target cells to undergo specific lysis in the presence of effector lymphocytes.

Three types of effector cells were analyzed: freshly harvested lymphocytes from tumor-bearers; TCGF-propagated lymphocytes (CTC); and specifically sensitized lymphocytes. Whereas the first two preparations are presumed to express natural killer (NK) activity [12–14], the latter is expected to react selectively with the sensitizing tumor cells.

In the murine M109 system, cultured tumor cells were slightly more effective stimulators than the fresh tumor cells and the optimum responder/stimulator cell ratios were higher (200–800/l with the cultured cells, compared with 20–100/l with fresh tumor cells); however, both cell types showed a similar degree of spontaneous [^{51}Cr] release and specific lysis (data now shown). In the human system only fresh tumor cells were used for *in vitro* sensitization, and in this case 6-day cultured tumor cells were significantly more sensitive to killing by stimulated autologous lymphocytes than were the fresh-frozen autologous tumor cells (data not shown).

The findings shown in Table 4 indicate that a similar level of specific cytotoxicity with the three types of effector cells is obtained, whether targets were labeled for 1 or 16 hr. In these experiments targets labeled for 16 hr were used at 1×10^3, as compared with 1×10^4 cells labeled for 1 hr. In other preliminary experiments we found that carcinoma target cells labeled for 16 hr could be used successfully with only 200 cells per well because of their high counts. The experiments depicted in Table 4 also show that whereas the NK-sensitive nonadherent tumor cells (K562 and YAC-1) were readily lysed by unsensitized and sensitized lymphocytes in the short-term assay, the fresh and cultured carcinoma target cells required a longer incubation period to manifest levels of lysis. CTC were effective killers in both the short- and the long-term assays.

DISCUSSION

This paper describes a rapid and simple technique for the isolation from human and mouse carcinomas of viable tumor cells of high purity and their use in the induction and assessment *in vitro* of cell-mediated cytotoxic response. Techniques currently employed for the isolation of tumor cells and the *in situ* effector cells (lymphocytes and macrophages) are based on a relatively long exposure of the tumor tissue to enzyme mixtures followed by various cell fractionation procedures, mainly velocity, isokinetic or isopycnic sedimentation [1–3, 15, 16]. These techniques are time-consuming and sometimes require special equipment and expertise. Furthermore, in the course of the lengthy isolation procedures, tumor cells may be damaged or lose important immunogenic determinants.

In the present technique, which may be generally applicable to a range of malignancies beyond those employed here, brief exposure to the mixture of enzymes (trypsin, collagenase, DNase) allowed subsequent efficient and easy dispersion by mechanical means, and at the same time eliminated dead cells. Fractions enriched in viable tumor cells were obtained by a simple gradient separation and, with most tumors, the whole procedure was completed in less than 1 hr. The same procedure might also be utilized for isolation of tumor-infiltrating effector cells by appropriate modifications of the gradient, i.e. additions of one or two more layers of Percoll or BSA with intermediate densities.

Tumor cells obtained by this technique were capable of (a) growing in culture, (b) forming tumors in mice, (c) stimulating cytotoxic responses *in vitro* and (d) serving as adequate target cells *in vitro* in the [^{51}Cr] release assay. Cryopreserved mouse and human tumor cells retained all these functions.

Measurement of cell-mediated cytotoxicity *in vitro* against fresh and cultured carcinoma cells was facilitated by several modifications of the

*Table 4. Cytotoxic activity of human and mouse lymphocytes measured by short and long-term [^{51}Cr] release assays using target cells labeled for 1 or 16 hr**

Effector cells†	Target cells	Mean % [^{51}Cr] release at:‡ 4–6 hr		16–24 hr	
		Targets labeled for 1 hr	Targets labeled for 16 hr	Targets labeled for 1 hr	Targets labeled for 16 hr
Fresh unsensitized PBL	Autologous	2	2	5	4
Sensitized PBL	Lung carcinoma (fresh-frozen)	13	15	24	29
Fresh unsensitized PBL	SK-MES 1	12	15	28	30
CTC		37	41	77	83
Fresh unsensitized PBL	MCF7	8	10	26	29
CTC		31	36	81	77
Fresh unsensitized PBL	K562	35	37	68	67
CTC		49	51	77	82
Fresh unsensitized splenocytes	M109 (fresh)	2	2	4	5
Sensitized splenocytes		21	18	59	54
CTC		47	52	86	91
Fresh unsensitized splenocytes	Mammary tumor (fresh)	2	3	5	6
CTC		27	26	47	48
Fresh unsensitized splenocytes	YAC-1	23	25	47	53
CTC		48	55	88	90

*Target cells labeled, washed and 'rested' for 3 hr in the presence of 10% FBS. Targets labeled for 1 hr were used at 1×10^4 cells per well; targets labeled for 16 hr were used at 1×10^3 per well. The effector/target cell ratio was 20/1. The human lung carcinoma cells were stored cryopreserved for 6 days. The M109 and the mammary tumor cells were freshly harvested.

†Human PBL and BALB/c mouse splenocytes were obtained from tumor-bearing hosts (lung carcinoma and M109 respectively) and tested before and after sensitization for 6 days in MLTC. CTC were obtained by culturing the lymphocytes for 1 month (human) or 3 months (mouse) in TCGF-containing conditioned medium.

‡Results are means of 4 separate experiments. Percentage of [^{51}Cr] released from target cells incubated alone was subtracted.

[^{51}Cr] release assay. Target cell labeling for 16–18 hr followed by washing and 'resting' in serum-containing medium allowed the use of highly labeled target cells in small numbers and the extension of the assay to 24 hr, without the limitation of excessively high spontaneous release of the label. Although other methods for long-term cytotoxicity assays are available, such as terminal labeling [17] or prelabeling with [^{111}indium] oxine [18], they are either more complicated to perform or are limited by the short half-life of the isotope respectively.

The cytotoxicity experiments conducted herein showed that unsensitized fresh human lymphocytes have nonspecific cytotoxic activity against both non adherent and adherent tumor cell lines but not against autologous fresh tumor cells, and this activity was more evident in the long-term assay (Table 4). Freshly obtained mouse splenocytes demonstrated natural cytotoxic activity against the YAC-1 lymphoma but had little or no detectable cytotoxicity against the solid tumors. In contrast, CTC obtained by culturing human and mouse lymphocytes in crude TCGF manifested strong cytotoxicity against all target cells, both fresh and cultured, lymphoid and solid, suggesting that they are activated NK cells [13, 14].

Lymphocytes specifically sensitized to murine syngeneic or human autologous carcinomas cytolysed the stimulating tumor cells (Table 4) and in addition expressed elevated NK activity (observations to be published).

In conclusion, it is hoped that the procedures

outlined in this work will encourage more studies with fresh solid neoplasms and may facilitate studies with primary autochthonous tumors.

Acknowledgements—We thank Dr. Grace Cannon of Litton Bionetics, Kensington, MD, for providing the human carcinoma cell lines, and Dr. James Reid, Dapartment of Hematology, Portsmouth Naval Medical Center, Portsmouth, VA, for some of the tumor biopsies and patients' blood.

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