

CLONAL ANALYSIS OF CYTOTOXIC T CELL RESPONSE
AGAINST HUMAN MELANOMA*

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The technology of cloning functional T cell subsets in vitro has proved to be a powerful tool in studies of cellular immune response to conventional as well as cell surface-associated antigens. Considering the scope of the technology in clonal analysis of cytotoxic immune response against autologous tumor cells, this study was undertaken to examine whether cytotoxic T lymphocyte (CTL) clones can be generated against autologous tumor cells in a human melanoma system and, if feasible, to employ such clones in assays designed to determine putative cell-mediated and/or circulating serum factor(s) capable of modulating cytotoxic function of such clones. We report in this communication that it is feasible to generate CTL clones with specificity directed toward an autologous human melanoma cell line, and show that the cytotoxicity of the CTL clone against the autologous melanoma cells could not be modulated in microcytotoxicity assays in the presence of unfractionated autologous lymphocytes or serum.

Materials and Methods

Target Cells. The human tumor cell line VIP was derived from a primary melanoma. This cell line grew as an adherent culture, was not contact inhibited, expressed melanosomes, did not contain any virus-like particles, and produced tumors histologically consistent with melanoma in nude mice when injected subcutaneously at $\sim 0.5-1 \times 10^6$ cells inocula. The VIP-F:T line was originally established from normal skin from the same patient (derived from a site distant from the primary site). After growing well for several months as contact-inhibited fibroblasts, the VIP-F:T culture developed features characteristic of in vitro transformation (disorganization in the arrangement of the cells, fusion between cells, cell surface shedding, and marked tendency for anchorage-independent growth). Electron microscopic examination showed unidentifiable cytoplasmic granules without suggestion of definitive viral structures and no evidence of virus budding. When injected subcutaneously in nude mice at $0.5-1 \times 10^6$ cell inocula, these cells also produced a tumor whose morphology was consistent with that of a sarcoma. These two cell lines, and several other cell lines grown in our laboratory or obtained from other laboratories, were used. Tissue culture technique has been published previously (1).

Effector Cells. Blood mononuclear cells (referred to henceforth as unfractionated lymphocyte [Ly]) were isolated from peripheral blood on Ficoll-Hypaque gradient (1). Monocytes/macrophages were removed whenever needed by adherence on plastic flasks for 2 h and/or by nylon column incubation (1-3).

Phenotypic Analysis. Phenotypic analyses of Ly were performed by immunofluorescence

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technique using monoclonal reagents (monoclonal antibodies OKT3, OKT4, OKT8, and M-1 were obtained from Ortho Pharmaceutical Corp., Raritan, NJ; monoclonal antibody HNK-1 [Leu-7] was obtained from Becton, Dickinson & Co., Sunnyvale, CA). The stained cells were analyzed either in a fluorescence-activated cell sorter (FACS IV; B-D FACS Systems, Sunnyvale, CA) or examined under a microscope.

Interleukin 2 (IL-2). IL-2 was generated in mixed Ly culture (MLC) using the basic method of Mier and Gallo (4) with some modifications. Multiple allogeneic mononuclear cell fractions isolated on Ficoll-Hypaque gradients and spleen cells from a single donor were used in the MLC. The cultures were carried out at a concentration of 1×10^6 cells/ml in 500–1,000 ml vol in RPMI 1640, 10% human albumin, and 10 μ g/ml phytohemagglutinin-P (PHA-P; Difco Laboratories, Detroit, MI) at 37°C in 50% CO₂ for 72 h. The cell-free supernatants were concentrated down to 1/5 vol by absorption in Aquacide II (Calbiochem-Behring Corporation, San Diego, CA) in 3,500 mol wt dialysis Spectrapor membrane (Spectran Medical Industries, Inc., Los Angeles, CA). The concentrated supernatant (referred to henceforth as IL-2) was filtered through 0.22 μ m Millex filters (Millipore Corp., Bedford, MA), and aliquots were stored frozen at –20°C.

Generation of Cytotoxic Lymphocytes (CL). CL were generated against the VIP melanoma cells in a one-way mixed lymphocyte-tumor cell interaction (MLTI). $\sim 2 \times 10^7$ mononuclear cells isolated on Ficoll-Hypaque gradient were co-cultured with 0.5×10^5 VIP cells (irradiated with 4,500 rad from a cesium source), seeded the day before in 5 ml of Ham's F-10 medium containing 10% fetal calf serum (FCS) and antibiotics. On the 3rd d, an additional 3 ml of medium containing 10% FCS and antibiotics were added. On the 7th d, the sensitized lymphocytes were washed, refractionated on Ficoll-Hypaque gradient, and cultured in Ham's F-10 medium containing 10% FCS, antibiotics, and 10% IL-2. The cultures expanded rapidly thereafter and were fed with IL-2-containing medium three times a week.

Generation of CL Clones. Cloning of CL was performed by limiting dilution in 96-well microtiter plates (Costar, Cambridge, MA). Usually 200–300 wells were seeded. Each well was preseeded with 1×10^5 irradiated (4,500 rad) allogeneic mononuclear cells derived from a pool of four normal volunteers. Thoroughly washed and vigorously suspended CL were counted several times before seeding at various cell numbers in 20% IL-2. Wells were checked for colonies daily from the 6th d. Each well was fed with 20% IL-2-containing medium every 3rd d thereafter. Growing colonies were transferred to a new 96-well plate and expanded serially until 5–10 wells were grown, at which point the colonies were pooled in 2-ml wells. Growing colonies were expanded serially thereafter at a cell density of $\sim 3\text{--}5 \times 10^5$ cells per 2-ml well. Aliquots were frozen at –80°C in 10% DMSO and transferred to liquid nitrogen 24 h later. Colonies could be recovered without loss of function. Colonies maintained in continuous culture were restimulated every 2 wk with a 10-fold excess of irradiated allogeneic spleen cells and sensitizing autologous VIP melanoma cells. The clones described in this communication have been propagated >2 mo in culture.

Cell-mediated Microcytotoxicity (CMC) Assay. The basic ⁵¹Cr-release assay (5) was used. Briefly, thoroughly washed target cells were labeled with 0.1 ml of Na₂ ⁵¹CrO₄ (1 mCi/ml, specific activity 200 or more μ Ci/mg) in a 37°C bath for 30 min with periodic shaking. Thereafter, the cells were washed again, resuspended in 1 ml of medium, and placed in the same 37°C bath for another 30 min. The cells were then washed five times in medium and counted. Cell concentration was adjusted and $\sim 1,000\text{--}2,000$ labeled target cells were seeded in V-shaped microwell plates (Costar) in 0.1 ml of complete medium. Effector cells at desired concentrations were added in 3–5 replicates at different effector/target (E/T) ratios. The plates were centrifuged at 200 g for 2 min and incubated at 37°C for 4 h. To determine maximum release, 0.1 ml of 1% sodium dodecyl sulfate solution was added to the appropriate wells. The plates were centrifuged again at 400 g for 5 min. Aliquots containing 0.1 ml of samples were withdrawn in 1 ml of Hydrofluor and counted for activity in a liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). Percent specific lysis was determined by the following formula: [(experimental release – spontaneous release)/(maximum release – spontaneous release)] $\times 100$.

Results and Discussion

Fig. 1 shows a representative CMC assay with the unfractionated autologous Ly (macrophages removed by nylon column incubation) against the two autologous cell lines and the natural killer (NK)-sensitive cell line K562. Little cytotoxic activity was detectable with the unfractionated Ly against the transformed fibroblasts, and only minimal to modest levels of cytotoxicity were seen against the melanoma cells. The same Ly were, however, quite cytotoxic against the K562 cells.

Table I shows the activities of the CL generated in vitro in MLTI against the VIP melanoma cells followed by expansion in IL-2 for 2 wk. As can be seen, marked cytotoxic activity was generated against the sensitizing autologous target. It is noteworthy that the same Ly showed statistically significant (although considerably less) activity against the transformed fibroblasts. The K562 cells and another allogeneic melanoma cell line were lysed with considerable efficiency. In addition to these allogeneic cells, several other allogeneic targets were also lysed (individual data not shown). A variety of cell-mediated cytolytic phenomena, such as anomalous killing (6), lectin-activated cytolysis (7, 8), interferon-induced killing (9), or lymphokine-activated cytotoxicity (10), could explain this polyclonal generation of CL.

CL generated against the autologous VIP melanoma cells and expanded in IL-2 for 2 wk were cloned by limiting dilution technique. Fig. 2 shows one of several

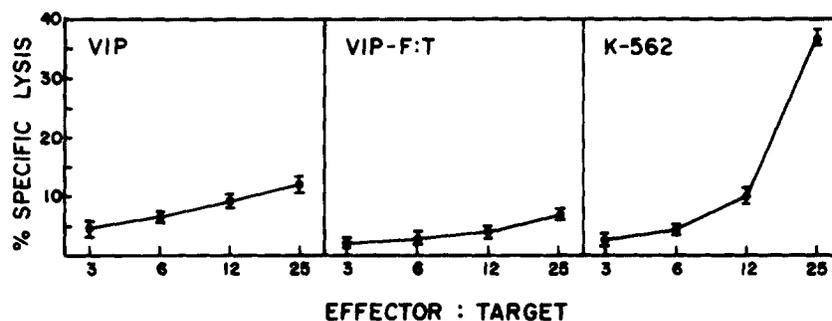


FIGURE 1. Cytotoxicity of unfractionated Ly against the two autologous targets and K562 cells. Each point represents percent specific lysis calculated from the mean of three replicate determinations.

TABLE I
Cytotoxicity of Unfractionated CL Generated in Autologous MLTI
against VIP Cells

Target	Percent specific lysis		
	E/T ratio		
	12:1	25:1	50:1
VIP	22*	34	58 (10) [†]
VIP-F:T	13	17	25 (8)
K562	14	27	36 (6)
MG-E (allogeneic melanoma)	24	31	40 (9)

* Data expressed as mean percent specific lysis of ⁵¹Cr-labeled target cells above medium (spontaneous release).

[†] Spontaneous release with individual targets.

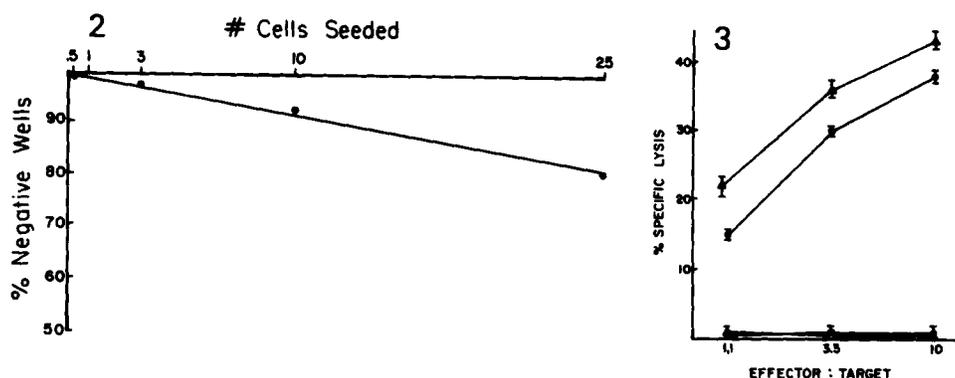


FIGURE 2. Data points with 0.5- and 1-cell wells are derived from 100 wells each; 3-, 10-, and 25-cell wells are derived from 32 wells each.

FIGURE 3. Cytotoxicity of clones E.5 and 3:1 against the autologous targets. (▲) E.5 against VIP; (Δ) E.5 against VIP-F:T; (●) 3:1 against VIP; (○) 3:1 against VIP-F:T.

limiting dilution cloning experiments. Several colonies were grown from wells seeded with 0.5–3 cells per well. Of these, two colonies (E.5, derived from a well seeded with 0.5 cells, and 3:1, derived from a well seeded with 3 cells) proved phenotypically homogeneous (bearing OKT8 phenotype exclusively) and expressed cytotoxicity selectively against the autologous target VIP cells only. Fig. 3 shows the cytotoxicity of the colonies against the two autologous targets. Both colonies were highly cytotoxic against the VIP melanoma cells, whereas the transformed fibroblasts were not lysed. It is noteworthy that the parent CL line from which these colonies were generated expressed statistically significant levels of cytotoxicity against the transformed fibroblasts (Table I). Detailed phenotypic analyses revealed no OKT4, M-1, or HNK-1 phenotypes within either colony. Colony E.5 was 99% positive and colony 3:1 was 98% positive for OKT8 phenotype.

The range of cytotoxicity and the specificity screen of the colonies are shown in Fig. 4. The activities of both colonies were restricted to the autologous melanoma cells, and the cytotoxic profile of the two colonies was remarkably identical. Although colony 3:1 was derived from a three cell-seeded well, the phenotypic homogeneity and, most importantly, the selective cytotoxic activity of the colony (restricted solely to the sensitizing autologous melanoma cells) suggested that it was quite possibly a true clone. The phenotypic homogeneity and the highly selective cytotoxic activity of colony E.5 were also suggestive of a true clone. The target cell panel used in the specificity screen has been reasonably large and included several NK-sensitive and other allogeneic targets against which the unfractionated CL showed significant cytotoxicity. This restricted expression of cytotoxicity therefore argued against anomalous killing, lectin-dependent cytolysis, or lymphokine-mediated killing. Taking this into consideration, these two colonies will henceforth be referred to as clones.

Presently, no conclusion can be drawn as to whether these two clones expressed any fine specificity directed against a putative tumor-associated antigen, since the very nature of the tumor antigen remains unclear. Such a possibility, however,

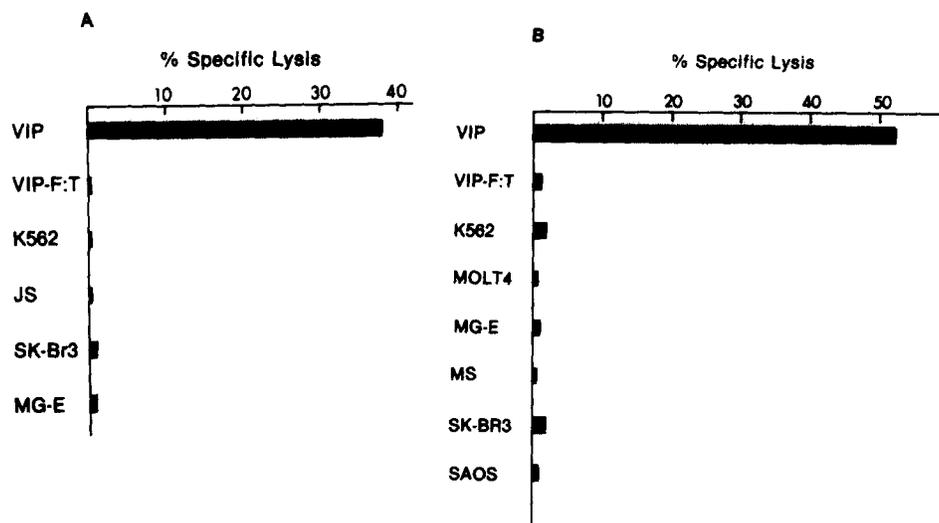


FIGURE 4. Range of cytotoxic activity of clones 3:1 (A) and E.5 (B). In addition to the two autologous targets, the target panel consists of the following: K562 and MOLT4 (NK-sensitive targets); JS, MG-E, and MS (allogeneic melanoma cells); SK-BR3 (human breast cancer line); SAOS (human sarcoma line). E/T ratios = 10:1.

TABLE II
*Cytotoxicity of Clone E.5 against VIP Cells in the Presence of
Autologous Serum and Unfractionated Ly*

In the presence of:	Percent specific lysis at E/T = 10:1
Medium alone	40*
Autologous serum [‡]	38
AB serum [‡]	37
Autologous Ly [§]	
Ly/E.5 = 1.25:1	41
2.5:1	39
5:1	43

* Differences in percent specific lysis were not statistically significant (Student's *t* test). Spontaneous release was 6%.

[‡] Autologous and AB-positive serum were added in 50% concentration.

[§] Viability of the frozen cells was >95%.

cannot be ruled out. Of the two clones, clone E.5 was expanded by repeated restimulation against irradiated VIP cells and allogeneic spleen cells. Experiments were therefore designed with this clone to search for putative autologous cell- or serum-mediated modulating factors. Table II shows the results of an experiment carried out to examine the effect of the unfractionated lymphocytes (collected before surgical removal of the melanoma from the patient and kept frozen at -180°C) as well as to search for any circulating factor(s) in the serum potentially capable of modulating the cytotoxicity of the CTL clone. As can be seen, no abrogation of cytotoxicity was detected with the unfractionated Ly or with the autologous serum obtained at the same time. While it will not be possible to draw any conclusion as to whether or not the generation of cytotoxic immune response in this particular case was controlled in vivo by some as yet unknown

mechanism, this study clearly suggests that analysis of cellular immune response against autologous tumor might be feasible with autoreactive clones generated by *in vitro* cloning technology.

Summary

We investigated the feasibility of generating cytotoxic T cell clones against autologous human melanoma cells using a melanoma cell line (VIP) and a spontaneously transformed autologous fibroblast line (VIP-F:T). Cytotoxic lymphocytes (CL) generated against the VIP melanoma cells in one-way mixed lymphocyte-tumor cell interactions were expanded in interleukin 2 for 2 wk. The expanded CL were cloned in limiting dilution. Two phenotypically homogeneous clones (3:1 and E.5) were obtained bearing OKT3 phenotype. Both clones expressed cytotoxicity selectively only against the sensitizing autologous target VIP. Cytotoxicity assays performed with clone E.5 against the VIP target cells in the presence of autologous unfractionated lymphocytes or serum showed no modulation of autoreactivity of clone E.5. These results indicate that analysis of cellular immune response against autologous tumor cells might be feasible using autoreactive clones generated by the currently available *in vitro* cloning technology.

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