

CYTOTOXIC T CELLS BOTH PRODUCE
AND RESPOND TO INTERLEUKIN 2

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Interleukin 2 (IL-2) is a T cell-derived lymphokine that serves as a cofactor for the in vitro response of T lymphocytes to antigen and plays an important role in regulating the growth and/or differentiation of these cells (1, 2). It has been postulated (2, 3) that IL-2 is produced by a discrete regulatory T cell subset, with its effects being exerted on a second, functionally distinct subpopulation of T cells. Cytotoxic T cells have been included in the IL-2-responsive subset (3). Several models of immune regulation have further assumed that the T lymphocyte pool is divided into a complex array of genetically preprogrammed T cell subtypes, each performing a specific regulatory or effector function (4, 5). However, recent results from several laboratories (6-8) have failed to support such a strict functional subdivision of the T cell pool.

The availability of highly purified mouse IL-2 (1) prompted us to reevaluate the distinction, if any, between IL-2-producing and IL-2-responsive T cells. For this purpose, we resorted to a cell-cloning procedure using activated T lymphocytes that were maintained only for short periods in culture. T cell clones were tested for cytotoxic activity, responsiveness to IL-2, and for the capacity to produce IL-2 after appropriate stimulation. We found no evidence for the existence of a major functional subdivision involving these parameters among alloantigen-activated T cells: the majority of clones analyzed could perform all three functions.

Materials and Methods

Mice and Cell Lines. Male C57BL/6J (H-2^b) and BALB/c (H-2^d) mice from The Jackson Laboratory, Bar Harbor, ME were used at 8-12 wk of age. Outbred Swiss white mice (NCS) were bred at The Rockefeller University and used when 6-7 wk old. The P815 mastocytoma (H-2^d) was maintained by weekly passage in cell culture (9).

T Cell Activation and Cloning. Mixed lymphocyte cultures were established by mixing 10⁶ C57BL/6J lymph node cells with 1.5 × 10⁶ BALB/c splenic stimulator cells in multiwell plates (76-033-05; Linbro Chemical Co., Hamden, CT). The stimulator cells were previously irradiated with 1,000 rad from a ⁶⁰Co source. Cultures were maintained in 1 ml of Eagle's minimal essential medium (EMEM) (410-1500; Gibco Laboratories, Grand Island, NY) containing 5 or 10% vol/vol heat-inactivated fetal calf serum (HIFCS) (Flow Laboratories, Inc., McLean, VA), 0.1 mM B-mercaptoethanol (B-ME), 26 mM sodium bicarbonate, and 5 μg/ml gentamycin sulfate (Sigma Chemical Co., St. Louis, MO). This medium will be referred to as "complete medium". Cultures were then incubated in a humidified atmosphere of 10% CO₂/7% O₂/83% N₂ for 4 d. To obtain a cell population suitable for cloning, we grew cultures of alloantigen-activated T cells using

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the conditioned medium from concanavalin A (Con A)-activated NCS spleen cells (CM) as a growth factor (10). About 10^5 viable cells from 4-d mixed lymphocyte cultures were transferred to 5 ml of complete medium containing 10% vol/vol CM and incubated for a further 3 d. At this stage, the expanded cell populations ($\sim 10^6$ cells/ml) were 99% viable and contained >99% T cells as assessed by susceptibility to lysis with Thy-1 antibody plus complement (10). For cloning experiments, 2×10^5 T cells were suspended in 1 ml of complete medium and $0.5 \mu\text{l}$ of the cell suspension was transferred via micropipette to microtiter wells (Linbro 76-203-05). The number of cells in each droplet was then determined by observation with an inverted microscope. Droplets containing single cells were supplemented with $100 \mu\text{l}$ of complete medium containing 10% vol/vol CM and 5×10^5 gamma-irradiated (2,000 rad) BALB/c spleen cells, and incubated at 37°C as described above. After 2 d, each well was fed with $100 \mu\text{l}$ of complete medium containing 10% vol/vol CM: every 2–3 d thereafter, half the medium was removed and replaced with an equivalent volume of fresh complete medium plus CM. Clones of rapidly growing T cells became apparent within 4–5 d of culture. After 7–10 d, positive and negative wells were scored, and colonies derived from a single parent cell were transferred to 24-well plates (Linbro 76-033-05) containing 1 ml of complete medium plus CM and 5×10^6 gamma-irradiated (2,000 rad) BALB/c spleen cells. As positive and negative controls, colonies derived from wells that were seeded with 100 or with no parent cells were also transferred. After 2 d, each well was fed with 1 ml of complete medium plus CM: every 2–3 d thereafter half the medium was removed and replaced with fresh medium plus CM. At about weekly intervals, the cultures were also supplemented with 5×10^6 gamma-irradiated (2,000 rad) BALB/c spleen cells. Assays for T cell function were performed 5–10 d after the last addition of feeder cells.

Cytotoxic Assay. Cytotoxic activity was assayed with ^{51}Cr -labeled P815 target cells using a modification of a procedure previously described (11). Targets (5×10^6 /ml in EMEM plus 2.5% vol/vol HIFCS) were labeled by addition of $500 \mu\text{Ci/ml}$ $\text{Na}^{51}\text{CrO}_4$ (350–600 mCi/mg chromium; Amersham Corp., Arlington Heights, IL). After incubation for 1.5 h at 37°C the targets were washed and resuspended at 5×10^4 /ml in EMEM plus 5% vol/vol HIFCS. Triplicate $50\text{-}\mu\text{l}$ samples of the target cell suspension were each mixed with $50 \mu\text{l}$ of the effector cell suspension (1×10^5 /ml) in round-bottomed microtiter trays (Linbro 76-013-15) and incubated in a humidified atmosphere of 10% CO_2 in air for 4 h. The extent of lysis was determined by counting $50\text{-}\mu\text{l}$ samples of the culture supernatant for 5 min in a gamma scintillation spectrometer. Results are expressed as \log_{10} cytotoxic units (CU) per assay well (11). Triplicate assays of cytotoxic activity have a standard deviation of $\pm 0.2 \log_{10}$ CU.

Response of T Cell Clones to IL-2. T cell clones were washed and resuspended at 10^3 viable cells/ml in complete medium. The response of the clones to IL-2 was determined by mixing $25 \mu\text{l}$ of the cell suspension with either $25 \mu\text{l}$ of medium alone or $25 \mu\text{l}$ of medium containing 50–100 U of IL-2 activity (1). Crude CM or highly purified IL-2 were used as a source of IL-2 activity. The cultures were set up in microtiter plates (Linbro 76-033-05) and incubated for 16–20 h in a humidified atmosphere of 10% CO_2 in air. IL-2-induced T cell proliferation was measured by incorporation of [^3H]methyl thymidine as described (12).

Induction of IL-2 Release by T Cell Clones. Alloantigen-activated T cells fail to produce detectable levels of IL-2 unless they are triggered by a secondary stimulus with either mitogen or specific alloantigen (10). The remaining cells in each clone (between 10^5 and 10^6 cells) were resuspended in 1 ml of medium (EMEM plus 0.1 mM B-ME) and lymphokine release was triggered by either (a) addition of $5 \mu\text{g/ml}$ Con A (grade IV; Sigma Chemical Co.) (after 2 h, the cell monolayers were washed with EMEM to remove unbound Con A and replenished with 1 ml of fresh medium); or (b) addition of 10^6 UV-irradiated P815 cells (irradiated in a 1-mm-deep cell suspension with 50 joules/ m^2 from a germicidal lamp).

The cell-free supernatant media were collected 4–12 h after the addition of antigen or washing of the Con A monolayers; this time period has been shown (10) to be optimal for detecting IL-2 production by uncloned T cells. Cell extracts were also prepared from the cell pellets by three cycles of freeze-thawing in 0.1 ml of double-distilled deionized water.

T Cell Growth Factor (TCGF) Assay for IL-2. Lymphokine preparations were assayed for their capacity to maintain the proliferation of Con A-activated blast cells as described (12).

Purified IL-2. IL-2 was purified as described (1). Alternatively, highly purified IL-2 was prepared by passing CM through a Sepharose anti-IL-2 IgG column.¹

Results

Growth Characteristics of Cloned T Cells. Expanded populations of C57BL/6J anti-BALB/c-activated T cells were cloned in the presence of CM and gamma-irradiated BALB/c feeder spleen cells. Colonies of rapidly proliferating T cells were easily identified by light microscope examination 4–5 d after the cloning step. At 7–10 d of culture, the number of positive and negative wells was scored; the average efficiency of colony formation by culture wells seeded with a single parent T cell was 40%, ranging from 28 to 54% in seven separate cloning experiments. The efficiency of colony formation fell to 0% if either CM or feeder cells were omitted from the cloning procedure. It is interesting that colony formation was reduced to ~20% if feeder cells syngeneic to the responder, rather than the stimulator population, were used, possibly indicating that antigen recognition plays some role in maintaining the propagation of alloantigen-activated T cells.

During the first 12–28 d of culture, cloned T cells continued to proliferate with an average division time of 23 h (range of 11–37 h in five cloning experiments). After ~33 d, cell proliferation began to slow, and the clones eventually reached a “stationary phase” during which little or no cell growth occurred. This stationary period continued until the 8th or 9th wk after cloning and was succeeded by a period of rapid and apparently continuous cell proliferation (division time ~24 h). Similar growth patterns have been observed by other investigators (13) working with long-term cultures of murine lymphocytes and it seems likely that the rapid onset of cell multiplication that follows the stationary phase reflects transformation of cells within these populations. To avoid difficulties of interpreting data obtained from possibly transformed, abnormal cell populations, all functional analyses were performed within the first 12–33 d after cloning. A total of 46 clones was examined; these ranged in size from 2 to 12×10^5 cells.

Cytotoxic Activity of T Cell Clones. All of the 46 clones expressed significant levels of lytic activity in a conventional ⁵¹Cr release assay against P815 targets (Table I). The range of cytotoxic activities expressed was wide, but of the same order of magnitude expressed by positive control cultures derived from 100–200 parent cells. Negative control cultures (parallel cultures containing feeder cells alone) failed to express any detectable cytotoxicity.

Response of T Cells to IL-2. All 46 clones proliferated when cultured in the presence of TCGF activity, in the form of either crude CM or highly purified IL-2. Both lymphokine preparations gave similar results. Table II shows that IL-2 induced a proliferative response 5–30-fold over the background level expressed by cells cultured in the presence of medium alone. The range of stimulation was similar to that observed with cells derived from positive control cultures. Negative

¹ Granelli-Piperno, A., L. Andrus, and E. Reich. Effect of antibodies to interleukin 2 on in vitro an in vivo immune responses. Manuscript in preparation.

TABLE I
All of the 46 Clones Tested Expressed Cytotoxic Activity Against P815 Targets

Type of colony	n	Log ₁₀ CU/culture well	
		X ± SD	Range
Clones	46	3.4 ± 0.4	2.6–3.9
Positive controls	17	3.6 ± 0.5	2.6–4.3
Negative controls	13	<1.7	

TABLE II
All of the 46 Clones Tested Proliferated in Response to Added TCGF Activity

Type of colony	n	Stimulation index	
		X ± SD	Range
Clones	46	10.4 ± 4.6	3.6–25.7
Positive controls	10	13.9 ± 6.4	7.8–23.1
Negative controls	13	1.1 ± 0.2	0.8–1.4

TCGF activity: crude CM or purified IL-2. Stimulation index = test cpm divided by cpm in the presence of culture medium alone.

TABLE III
38 of the 46 T Cell Clones Tested Produced Detectable TCGF Activity

Type of colony	n	Range of TCGF production (Units activity/10 ⁵ cells)
Clones	38	24–210
Clones	8	—
Positive controls	10	37–201
Negative controls	13	—

Lymphokine production was triggered by Con A or antigen stimulation as described in Materials and Methods. These two methods of stimulation induced similar levels of TCGF activity and the results shown are a summary of these data.

control cultures showed no significant proliferation in response to added IL-2 (Table II).

TCGF Production by T Cell Clones. The remaining cells in each clone (between 10⁵ and 10⁶ T cells) were tested for their capacity to produce IL-2 activity after appropriate stimulation. Table III shows a summary of the results of this study. 38 of the 46 clones produced TCGF activity that could be detected in the supernatants and/or extracts of these cells (these two methods of lymphokine preparation gave similar results). The levels of TCGF activity produced by the clones were equivalent, based on cell number, to those produced by positive control cultures (Table III), and were comparable to the levels of activity produced by bulk cultures of mixed leukocyte culture-activated T cells (10) or by cloned helper cell lines (14). The possibility that feeder cells contribute to the TCGF activity produced can be excluded since negative control cultures containing irradiated feeder cells alone failed to generate detectable activity after incubation with either Con A or alloantigen (Table III).

Discussion

We have used a short-term culture technique to investigate the clonal relationship of IL-2-producing and IL-2-responsive T cells. The results showed that the majority of alloantigen-activated T cells were multifunctional, all were cytotoxic, and most possessed the capacity both to respond to IL-2 and to produce material with TCGF activity. Preliminary experiments using a potent rabbit antiserum raised against purified murine IL-2 have confirmed that the clone-derived TCGF activity is indeed IL-2 (manuscript in preparation). It appears therefore that the mature alloantigen-reactive T cell is not necessarily precommitted towards expression of either regulatory or effector functions. Recently, in a study similar to our own, Kelso and MacDonald (15) were unable to detect a high frequency of IL-2-producing cytotoxic cells responding to H-2 alloantigens. One possible reason for this discrepancy is the elaboration in T cell cultures of a potent inhibitor of IL-2 that appears 12 h after antigen stimulation and can completely mask IL-2 activity in the TCGF assay (10). In their study (15), lymphokine preparations were collected after the 12-h period. While it is clear that in our own study, most of the cloned cultures were polyfunctional, we have no proof that all individual cells in these clones are at all times expressing all functions: it is possible that the activated parent cells can differentiate along multiple pathways and that each clone contains a mixture of these. Alternatively, the three functions may represent partially or entirely distinct physiological states of a single cell type.

17% of the IL-2-responsive clones failed to produce detectable TCGF activity after appropriate stimulation. Whether these cells represent a truly functionally distinct T cell population, or whether this result simply reflects limitations in the system for detecting IL-2 production by small numbers of T cells is at present unclear. Several groups have described T cell clones that express an apparently restricted repertoire of immunological functions (16, 17). However, these studies were performed with long-term (>3–12 mo) cultured T cells which undoubtedly express aneuploid karyotypes and are, perhaps, in addition, transformed. The predominant cell type obtained in our short-term study was multifunctional and we suggest that such cells are more representative of the normal T cell pool.

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