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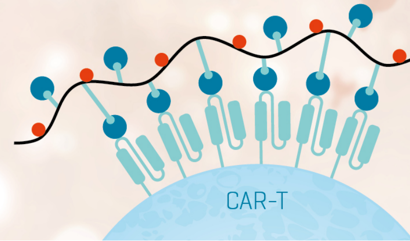

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BIOCHEMICAL AND BIOLOGIC CHARACTERIZATION OF LYMPHOCYTE REGULATORY MOLECULES

III. The Isolation and Phenotypic Characterization of Interleukin-2 Producing T Cell Lymphomas

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To isolate a stable tumor cell line source of Interleukin 2 (IL-2 formerly referred to as T cell growth factor), over 40 murine leukemia and lymphoma cells as well as 9 clonal helper and killer IL-2-driven T cell lines were screened for both constitutive and mitogen-stimulated IL-2 production. A radiation-induced splenic lymphoma from the B10.BR mouse, the LBRM-33 cell line, could be stimulated to produce over 1000 units/ml of IL-2 after 24 hr exposure to T cell mitogens. Peak IL-2 activity was found in supernatants harvested from 24-hr cultures of either 1% PHA or 20 μg/ml Con A-stimulated LBRM-33 cells (10^6 cells/ml). IL-2 production observed in both serum-free and serum-containing cultures represented between 1000 and 5000 times the quantity of IL-2 produced in conventional cultures of mitogen-activated rat or mouse spleen cells. Peak IL-2 production by LBRM-33 cultures (stimulated at either optimal Con A or PHA concentrations or co-stimulated with suboptimal amounts of mitogen and phorbol myristate acetate) was consistently accompanied by LBRM-33 cell death. Phenotypic characterization of the producer cell revealed LBRM-33 cells to be Thy 1+, Ly 1+, Ly 2+ Ly 3+, Qa 2-3+, Qa 3.2+, Qat 4+, and Ly 5+. These studies provide further evidence that IL-2 is a T cell product and establish a source of IL-2 that will be a valuable reagent for the isolation and further molecular characterization of this immunoregulatory molecule.

Recent studies conducted in several laboratories have confirmed that a soluble protein, T cell growth factor, (TCGF,[4] now referred to as Interleukin 2 or IL-2) is present in culture medium conditioned by T cell mitogen/antigen-stimulated mouse, rat, or human mononuclear leukocytes (1-8). As a potent immunoregulatory factor, purified IL-2 has been found to i) markedly enhance thymocyte mitogenesis, ii) induce cytotoxic T cell (CTL) generation in both alloantigen-stimulated thymocyte or nude spleen cell cultures, and iii) promote anti-erythrocyte (RBC) plaque-forming cell responses in red blood cell (RBC)-stimulated nude spleen cell cultures (6, 7). Furthermore, IL-2 has to date been found to be the only biologic mediator capable of promoting the sustained *in vitro* proliferation of antigen-specific helper or killer T cell lines (1, 2, 8).

Given its ability to enhance *in vitro* immune reactivity, the potential of IL-2 as an agent that could be used to control *in vivo* T cell immune responses is significant. However, to conduct meaningful *in vivo* manipulation experiments, large quantities of IL-2 are required. By using available preparative purification schemes (6, 7) it would be necessary to process literally hundreds of liters of conditioned medium. In hopes of establishing a means of producing conditioned medium with significantly higher IL-2 titers, we screened over 40 T cell leukemia and lymphoma cell lines for both constitutive and mitogen-induced IL-2 production. Screening trials also included testing of IL-2 growth dependent clonal killer and helper T cell lines (2-9). Of the cell lines tested only one, a radiation-induced splenic lymphoma from the B10.BR mouse, (LBRM-33), was found to produce high titers of TCGF upon mitogen stimulation. In fact, 1% phytohemaglutinin-A (PHA) stimulation of LBRM-33 cells resulted in culture supernatants that contained between 1000 and 5000 times the amount of biologically active IL-2 that is consistently produced by similar numbers of optimally mitogen-stimulated rat or mouse splenocytes. Subsequent factor production kinetics and mitogen-induced dose responses of LBRM-33-derived IL-2 (as well as the phenotypic characterization and fate of LBRM-33 cells upon TCGF production) provide additional hypotheses concerning the nature and response of naturally occurring IL-2 producer T cells.

MATERIALS AND METHODS

Screening of T cell lines for IL-2 production. In vitro and *in vivo* passaged T cell leukemia and lymphoma cell lines were collected from tissue culture sources at the Memorial Sloan

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[4] Abbreviations used in this paper: CTL, cytotoxic T lymphocyte; CTLL, cytotoxic T lymphocyte line; ^{3}H-Tdr, tritiated thymidine; IL-1, Interleukin-1; IL-2, Interleukin-2; LAF, lymphocyte activating factor; PMA, phorbol myristate acetate; RBC, red blood cell; TCGF, T cell growth factor.

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Kettering Cancer Center, New York, and the University of California at Irvine (see Table I). Single cell suspensions of each tumor line (1×10^6 cell/ml) were cultured in 200 μl flat bottom microplate wells (3596 microtiter plate, Costar Inc., Cambridge, MA) in RPMI 1640 supplemented with 10% heat inactivated (56°C for 30 min) fetal calf serum (FCS), 2.5×10^{-5} M 2-mercaptoethanol, 25 mM HEPES buffer, 50 U/ml penicillin, 50 μg/ml streptomycin, and 300 μg/ml fresh L-glutamine. Replicate microwell cultures were stimulated with either i) tissue culture medium, ii) Con A, 2.5 μg/ml, iii) PHA (1% by volume, Grand Island Biologicals Corp., Grand Island, NY), or iv) *Escherichia coli* lipopolysaccharide (100 μg/ml, Difco Inc., Detroit, MI). After 24, 48, and 72 hr, culture supernatants from all microwells were harvested and tested for IL-2 activity in a standard microassay based upon the IL-2-dependent replication of an antigen-specific cytotoxic T cell line (5).

In addition, several mono- and polyclonal helper and killer T cell lines (see Table I) were harvested from long-term IL-2 dependent culture, washed repeatedly, and stimulated in microwell cultures (10^6 cells/ml) with either FCS-supplemented RPMI 1640 medium or mitogen. After 24 hr of culture, harvested supernatants were tested for IL-2 activity. Helper T cell lines tested included HT-1, HT-2, and HT-3.1. The erythrocyte antigen specificities and mouse strain backgrounds of these clonal T cell lines have been previously described (2). Killer T cell lines tested included CTLL-1 and CTLL-2 as well as clonal derivatives CTLL-2 Clone 8, Clone 19, Clone 13, and Clone 121. The lytic specificities of these anti-tumor and anti-H-2^d killer T cell lines have been previously demonstrated (1, 9). We also screened CTLL 1-6 and 1-7 for IL-2 production. These clonal cell lines were derived from C57BL/6 mouse spleen cells originally stimulated by irradiated B6.CA (H-2^d lytic specificity, CTLL 1-6) or BALB/c (H-2^d lytic specificity, CTLL-1-7) splenocytes. Both CTLL 1-6 and 1-7 have been in continuous IL-2 dependent culture for over 12 mo and routinely mediate 60% specific lysis of appropriate ^{51}Cr-labeled (H-2^d) target cells at an effector/target cell ratio of 10:1. It was inappropriate to culture IL-2 dependent cytotoxic or helper T cells for periods longer than 24 hr, in that it has been repeatedly observed that in the absence of IL-2 such cells die within 24 hr of culture (5-7).

IL-2 producer cell cloning. Both LBRM-33 and RBL-3 cell lines were subcloned by limiting dilution in flat bottom microplate wells. Cells were seeded in 200 μl volumes of FCS supplemented RPMI 1640 at a concentration of 0.1 cell/ml. After 10 days of culture, microplate wells were screened for viable cell growth. Positive wells were harvested and subcultured in 25 cm^3 tissue culture flasks (3013, Falcon Plastics, Oxnard, CA) in FCS-supplemented RPMI 1640; 50 to 70% plating efficiency was observed in cloning trials of both RBL-3 and LBRM-33. Once the subcultures reached sufficient densities (10^6 cells/ml), the clonal cultures were harvested and retested for both constitutive and mitogen-induced IL-2 production as detailed above.

LBRM-33 IL-2 production characteristics. To determine optimal culture duration for stimulating LBRM-33 cells to produce IL-2, the cells were activated with 1% PHA in identical cultures for either 2, 4, 6, 12, 24, or 48 hr. At the end of the time periods indicated, culture supernatants were harvested and assayed for IL-2 activity. Similarly, in experiments designed to test optimal cell concentration for IL-2 production, LBRM-33 cells were cultured for 24 hr at various cell concentrations (ranging from 7.5×10^4 to 1×10^7 cells/ml) with 1% PHA, before supernatant IL-2 testing. Peak mitogen concentration for induction of LBRM-33 IL-2 production was assessed in identical cultures (10^6 cells/ml) stimulated with either Con A ($\log_2$ dilutions ranging from 2.5 to 160 μg/ml) or PHA ($\log_2$ dilutions ranging from 0.07 to 10% by volume). All production kinetics experiments were conducted by using both serum-containing and serum-free RPMI 1640.

In experiments designed to test the synergistic effects of mitogen and phorbol myristate acetate (PMA) upon LBRM-33-generated IL-2, PMA (1 to 50 ng/ml) was used in concert with 24 hr Con A and PHA stimulation over a wide mitogen concentration range (Con A: 10 to 60 μg/ml, PHA: 0.01 to 10% by volume). In some instances, to assess cellular proliferation, 24-hr mitogen-stimulated LBRM-33 cell cultures were harvested and percent trypan blue negative cells was counted. Alternatively, identical cultures were pulsed for 4 hr with tritiated thymidine (^{3}H-Tdr, 0.5 μCi/microplate well, 20 mCi/mM, New England Nuclear, Boston, MA), harvested with the aid of a multiple automated sample harvester and counted via liquid scintillation in a manner identical to that described below for assay of IL-2 activity.

Due to the well documented (10) involvement of cyclic nucleotide fluxes in cell activation, protein synthesis, and product secretion, we screened several pharmacologic agents for their ability to induce LBRM-33 IL-2 production. Details of these compounds (Sigma Chemical Corp., St. Louis, MO) and the concentrations tested are further delineated in the *Results* section. Thymopoietin (11) and its active *in vitro*-synthesized pentapeptide (TP5, 12) were generously provided by Dr. Gideon Goldstein, Ortho Pharmaceutical Corp., Raritan, NJ. Prostaglandins were obtained from the Upjohn Chemical Corporation, Kalamazoo, MI.

Assay for IL-2 activity. The amount of IL-2 activity present in supernatant samples was determined in a standard microassay (5) based upon the IL-2 dependent proliferation of CTLL-2, a cytotoxic T cell line (1). Briefly, CTLL cells were cultured in 200-μl volumes in flat bottom microplate wells in Clicks medium (Altick Associates, Hudson, WI) supplemented with 10% FCS, 300 μg/ml L-glutamine, 25 mM HEPES buffer, 16 mM $NaHCO_3$, 50 U/ml penicillin, and 50 μg/ml streptomycin. Each well contained 4000 CTLL cells together with a $\log_2$ dilution (ranging from 0.1 to 50% by volume) of a putative IL-2 containing sample. After a 24 hr incubation (37°C in a humidified atmosphere of 5% CO_2 and air) the microplate wells were pulsed with 0.5 μCi of ^{3}H-Tdr. Cultures were harvested 4 hr later onto glass fiber filter strips and ^{3}H-Tdr incorporation was determined via liquid scintillation. Results were quantified by probit analysis (5). TCGF activity was expressed in units/ml (U/ml) by comparing experimental probit data with that obtained from assay of a standard TCGF sample assigned a value of 1 U/ml (48 hr tissue culture medium conditioned by the 5 μg/ml Con A stimulation of rat splenocytes (10^6 cells/ml)).

Cell surface phenotyping. LBRM-33 cells were typed for the cell surface expression of Thy 1, LY, TL, and Qa markers. Phenotyping was conducted by using a direct antibody-protein A-sheep RBC rosetting assay, the methods for which have been previously described (13). Monoclonal anti-Thy 1.2, and conventional Ly 1.2, Ly2.2, and TL antisera was obtained from Dr. E. A. Boyse, Memorial Sloan Kettering Cancer Center. The methods of production and specificities of the conventional anti Ly and anti-Qa reagents used in typing studies have been previously described (14-16). To confirm Ly antigen expression of LBRM-33 cells, quantitative absorptions of anti-Ly 1.2, and anti-Ly 3.2 sera were conducted. Increasing concentrations of LBRM-33 cells (5×10^7, 1×10^8, 2×10^8 cells/ml) were incubated at room temperature in 100 μl of Ly antisera (1/200 dilution) for 1 hr. After absorption, serum samples were tested

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TABLE I

Screening of murine leukemia and lymphoma cells for IL-2 production

Cell Line	Strain of Origin	Inducing Agent	IL-2 Activity Present in 48-hr Supernatant after Activation with			
			Medium[1]	Con A[2]	PHA[3]	LPS[4]
			U/ml			
T Cell Tumors						
RBL-5	C57BL/6	RLV	0.0	0.0	0.0	0.0
EL-4	C57BL/6	Benzapyrene	0.0	0.0	0.0	0.0
CEL 41	C57BL/6	Subclone of EL-4	0.0	0.0	0.0	0.0
CEL 43	C57BL/6	Subclone of EL-4	0.0	0.0	0.0	0.0
CEL 49	C57BL/6	Subclone of EL-4	0.0	0.0	0.0	0.0
L51784	B10.129.5.M	Spontaneous	0.0	0.0	0.0	0.0
S49	BALB/c	Mineral oil	0.0	0.0	NT[5]	NT
BW5147	AKR	Spontaneous	0.0	0.0	NT	NT
RDM 4	AKR/I	Spontaneous	0.0	0.0	0.0	0.0
RBL-3	C57BL/5	RLV	0.0	0.0	0.08	0.0
RBL-31	C57BL/6	Subclone of RBL-3	0.06	0.56	0.31	0.0
RBL-3 IC	C57BL/6	Subclone of RBL-3	0.11	0.64	0.37	0.0
RBL-3M	C57BL/6	Subclone of RBL-3	0.0	0.0	0.0	0.0
YAC-1	A/SN	Mo-MuLV	0.0	0.0	0.0	0.0
TIMI	C57BL/6	Radiation	0.0	0.0	NT	NT
R1.1	BALB/c	Radiation	0.0	0.0	NT	NT
RAD-A1	A	Radiation	0.0	0.0	0.0	0.0
ASL-1	A	Spontaneous	0.0	0.0	0.0	0.0
RLδ-1	BALB/c	Radiation	0.0	0.0	0.0	0.0
HRST 34	hr/hr	Spontaneous	0.0	0.0	0.0	0.0
LBRM-33	B10.Br	Radiation	0.0	26.0	517.0	0.0
1A5	B10.Br	Subclone of LBRM-33	0.0	0.0	0.0	0.0
4C1	B10.Br	Subclone of LBRM-33	0.0	0.15	4.3	0.0
5A4	B10.Br	Subclone of LBRM-33	0.0	35.0	866.0	0.0
4A2	B10.Br	Subclone of LBRM-33	0.0	42.0	1163.0	0.0
6B1	B10.Br	Subclone of LBRM-33	0.0	32.0	927.0	0.0
B-Cell or Undifferentiated Tumors						
P3	BALB/c	Mineral oil	0.0	0.0	NT	NT
MPOC-11	BALB/c	Mineral oil	0.0	0.0	NT	NT
S194	BALB/c	Mineral oil	0.0	0.0	NT	NT
WEHI-22	BALB/c	Mineral oil	0.0	0.0	NT	NT
NS-1	BALB/c	Mineral oil	0.0	0.0	0.0	0.0
SP-2	BALB/c	Mineral oil	0.0	0.0	0.0	0.0
WEHI-7	BALB/c	Mineral oil	0.0	0.0	NT	NT
FBL-3	C57BL/6	FLV	0.0	0.0	0.0	0.0
MBL-2	Mo-MuLV	Mo-MuLV	0.0	0.0	0.0	0.0
L1210	DBA/2	Methylcholanthrene	0.0	0.0	0.0	0.0
Macrophage Tumors						
J774	BALB/c	Mineral oil	0.0	0.0	0.0	0.0
WEHI-3	BALB/c	Mineral oil	0.0	0.0	0.0	0.0
P38801	BALB/c	Mineral oil	0.0	0.0	0.0	0.0
IL-2 Dependent Effector T Cell Lines						
CTLL-1	C57BL/6	3-year-old killer T cell line	0.0	0.0	0.0	0.0
CTLL-2	C57BL/6	3-year-old killer T cell line	0.0	0.0	0.0	0.0
CTLL-2-clone 19-SKI	C57BL/6	Syngeneic tumor antigen reactive clone of CTLL-2	0.0	0.0	0.0	0.0
CTLL-2-clone 8-SKI	C57BL/6	Allo-antigen (H-2^d) reactive clone of CTLL-2	0.0	0.0	0.0	0.0
CTLL-2-clone 13-SKI	C57BL/6	Allo-antigen (H-2^d) reactive clone of CTLL-2	0.0	0.0	0.0	0.0
CTLL-2-clone 121-SKI	C57BL/6	Nonlytic clone of CTLL-2	0.0	0.0	0.0	0.0
HT1	C57BL/6	Sheep-RBC-specific helper T cell line	0.0	0.0	0.0	0.0
HT2	BALB/c	Sheep-RBC-specific helper T cell line	0.0	0.0	0.0	0.0
HT3.1	C57BL/6	Horse-RBC-specific helper T cell line	0.0	0.0	0.0	0.0
CTLL-16	C57BL/6	Allo-antigen (H-2^d) reactive killer T cell line	0.0	0.0	0.0	0.0
CTLL-17	C57BL/6	Allo-antigen (H-2^d) reactive killer T cell line	0.0	0.0	0.0	0.0

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TABLE I—*Continued*

Normal Lymphocyte Populations	IL-2 Activity Present in 48-hr Supernatant after Activation with			
	Medium[1]	Con A[2]	PHA[3]	LPS[4]
		U/ml		
Normal mouse splenocytes (10^6 cells/ml)	0.0	0.32	0.13	0.0
Normal mouse splenocytes (10^7 cells/ml)	0.0	1.13	0.87	0.0
Normal rat splenocytes (10^6 cells/ml)	0.0	1.0	0.86	0.0
Normal rat splenocytes (10^7 cells/ml)	0.0	13.6	7.85	0.0

[1] Click's 10% FCS.
[2] 5 μg/ml.
[3] 1% PHA-M by volume.
[4] 100 μg/ml.
[5] NT, not tested.

for residual cytolytic reactivity against C57BL/6 thymocytes in standard antibody dependent, C-mediated cytolysis assays (17). Reactivity was determined by trypan blue dye exclusion of treated thymocytes.

RESULTS

Screening of mouse leukemia and lymphoma cell lines for constitutive and lectin-induced IL-2 production. Table I details the derivation of the 40 murine tumor cell lines tested for IL-2 production. IL-2 activity present in 24 hr supernatants harvested from medium, Con A, PHA, and LPS-stimulated cultures is listed in terms of U/ml. As indicated, 48-hr tissue culture medium conditioned by Con A (5 μg/ml) stimulated rat splenocytes (1×10^6 cells/ml) contained 1 U/ml of IL-2 activity. Only two tumor cell lines were positive for IL-2 production. Of these, the radiation-induced splenic lymphoma, LBRM-33, generated 26 U/ml of IL-2 activity after Con A stimulation and 817 U/ml in PHA-stimulated cultures. However, LBRM-33 cells produced no IL-2 activity either constitutively or after LPS sensitization. The second cell line identified as an IL-2 producer ((RBL-3, a Thy 1 antigen positive (S. Gillis, unpublished observation)) Rauscher leukemia virus-induced lymphoma generated comparatively low levels of factor upon Con A (0.16 U/ml) or PHA (0.08 U/ml) stimulation. As observed with LBRM-33, RBL-3 produced no IL-2 either constitutively or after stimulation with LPS. Limiting dilution cloning of RBL-3 produced 26 viable sublines, three of which are detailed in Table I in terms of lectin-induced IL-2 production. Two RBL-3 sublines (K and I) produced 24 hr Con-A/PHA stimulated supernatants that contained approximately 3 to 4 times as much IL-2 as conditioned medium prepared in an identical fashion from the parent cell line. However, neither cell line generated as much IL-2 activity as mitogen-stimulated rat spleen cells.

One hundred clones of LBRM-3 were isolated by limiting dilution and screened for IL-2 production. Interestingly, of the clones harvested, only two were identified that did not produce high levels of IL-2. The IL-2 production capacities of six of the clonal derivatives of LBRM-33 are listed in Table I. It is interesting to note that in addition to defining one low producer (4C1) and one total nonproducer (1A5), limiting dilution cloning generated several sublines (5A4, 4A2, and 6B1, for example) that produced even greater quantities of IL-2 upon lectin stimulation than did the parent line, LBRM-33.

It is important to note that all clonal cytotoxic and helper T cell lines harvested from continuous IL-2 dependent culture were incapable of producing IL-2 following medium or lectin sensitization. Such an observation was not surprising, particularly upon considering the nature of long term cultures of effector T cells. Such cell lines have previously been shown to be acutely dependent for their proliferation upon the continual presence of exogenously supplied IL-2 (1–3, 5–9). Stimulation of these cells with mitogen alone is not sufficient to induce their proliferation (5). Conceivably if such a cell was capable of generating IL-2 (either constitutively or upon mitogen stimulation) it would be possible to grow that effector T cell line in the absence of IL-2 or in the presence of mitogen alone; which, as detailed elsewhere, is clearly not possible (1–3, 5–9).

It should be stressed that the IL-2 production data detailed in Table I were not different when supernatants were harvested from 48 or 72 hr medium or lectin-stimulated cultures (data not shown). Therefore, it is probable that the cell lines identified as negative for IL-2 production (with the concentrations of lectin indicated) do not yield negative values due to an alteration in IL-2 production kinetics.

LBRM-33 induced IL-2 production: kinetics and mitogen dose responses. As was clear from the data displayed in Table I, of the two IL-2 producer tumor lines identified, LBRM-33 (which upon PHA stimulation produced approximately 1000 to 5000 times the amount of factor generated by an equivalent amount of mouse or rat spleen cells) was by far the cell worthy of further investigation. To determine the production kinetics of LBRM-33-elicited IL-2, LBRM-33 cells were harvested from culture in FCS-supplemented RPMI 1640 and seeded in replicate microplate cultures at 10^6 cells/ml in the presence of 1% PHA. After 2, 4, 6, 12, 18, 24, and 48 hr of culture, supernatants were harvested and assayed for IL-2 activity. Figure 1 shows the results of these experiments conducted in both 10% FCS-supplemented and serumless RPMI 1640 medium. Regardless of the presence of serum, detectable IL-2 activity was first obtained between 5 and 7 hr after PHA stimulation. In both cases, peak levels of IL-2 were present in 16 to 24 hr culture supernatants (1000 to 1200 U/ml) and these titers diminished only slightly during a subsequent 24 hr culture. As shown in Figure 1, LBRM-33 IL-2 production kinetics were similar to those previously detailed for cultures of lectin stimulated rat, mouse, and human mononuclear leukocytes where measurable IL-2 activity was detected within 6 hr after mitogen stimulation (5). However, in addition to the magnitude of factor produced, LBRM-33 IL-2 production kinetics were somewhat different from normal production profiles in that the amount of IL-2 present in post peak cultures did not significantly decline over time. This is routinely observed in normal splenocyte IL-2 production as activated T cells absorb and use the IL-2 produced to foster their own proliferation (18–20). In a sense it was not surprising that post peak culture duration had little effect on IL-2 concentration since the LBRM-33 cells are not dependent on IL-2 for their own proliferation. In fact as detailed below, LBRM-33 cells in IL-2 production cultures are >95% trypan

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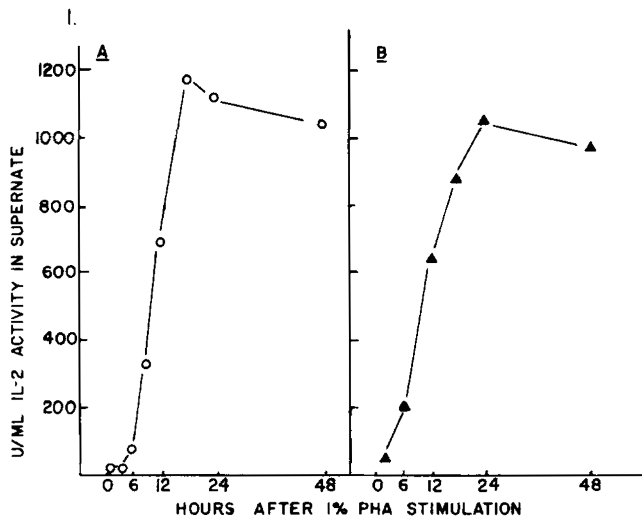

Figure 1. LBRM-33 IL-2 production kinetics. Supernatant IL-2 titers present in PHA-stimulated cultures conducted in the presence (*A*, ○——○) and absence (*B*, ▲——▲) of FCS.

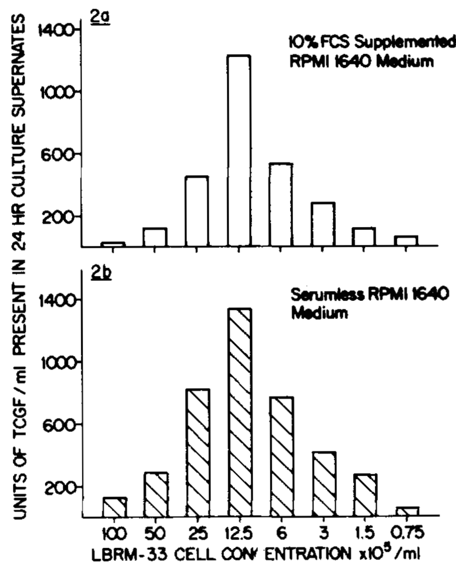

Figure 2. Twenty-four hr IL-2 production by 1% PHA-stimulated LBRM-33 cells, cultured at various cell concentrations in either FCS-supplemented (*A*) or serum-free (*B*) RPMI 1640.

blue positive after 24 hr stimulation with 1% PHA, indicating cell death.

Experiments similar to those displayed in Figure 1 were conducted in both serum-supplemented and serum-free conditions to determine optimal LBRM-33 cell concentration for IL-2 production. Based on the production kinetics detailed in Figure 1, supernatants were harvested from cultures containing varying concentrations of LBRM-33 cells (ranging from 10^5 to 10^7 cells/ml) 24 hr after stimulation with 1% PHA. As shown in Figure 2, optimal cell concentration for peak LBRM-33 IL-2 production was approximately 10^6 cells/ml.

Similar experiments were also conducted to determine optimal mitogen concentration required for LBRM-33 IL-2 production. Cells were plated in replicate microwell cultures (10^6 cells/ml both in the presence and absence of serum) and stimulated with a range of concentrations of PHA (0.1 to 10%). After 24 hr of culture, harvested supernatants were tested for IL-2 activity. As shown in Figure 3, peak IL-2 production (greater than 1400 U/ml) was observed in 1% PHA stimulated cultures irrespective of the presence of serum.

One of the most interesting observations of mitogen-induced LBRM-33 IL-2 production was that after 24 hr stimulation with PHA (and subsequent production of supernatant containing greater than 100 U/ml of activity) reculture of LBRM-33 cells could not be achieved. Furthermore, examination of the cultures revealed that 24 hr PHA-stimulated LBRM-33 cells were >95% trypan blue positive. To examine the correlation between mitogen sensitization, LBRM-33 cell survival, and IL-2 production, dose response experiments identical to those detailed in Figure 3 were conducted with Con A. In addition, 24 hr activated microwell cultures (stimulated with $\log_2$ concentrations of Con A ranging from 5 to 160 μg/ml) were either i) pulsed for 4 hr with ^{3}H-Tdr, harvested, and counted for thymidine incorporation or ii) harvested and scored for the percentage of trypan blue positive cells. Results of these experiments are diagramed in Figure 4. Once more we observed an inverse relationship between LBRM-33 cellular proliferation (as measured by ^{3}H-Tdr incorporation or viability) and IL-2 production. As was first observed in Table I, 5 μg/ml Con A sensitization of LBRM-33 cells (normally a peak mitogenic dose for rat and mouse splenocytes) resulted in the elaboration of only 20 U/ml of IL-2. Low IL-2 production was accompanied by high ^{3}H-Tdr incorporation and viability of stimulated cells. In contrast, cultures stimulated with Con A concentrations that triggered significant IL-2 production (20 μg/ml stimulation yielded supernatants containing 750 U/ml) were only 5% trypan blue positive and incorporated only background levels of ^{3}H-Tdr.

At present it is difficult to distinguish whether LBRM-33 cells die as result of IL-2 production or whether IL-2 is released as a result of mitogen toxicity. It is important to note that attempts to isolate active IL-2 from supernatants of nonstimulated LBRM-33 cells (via sonication) have been unsuccessful. Therefore it is questionable whether IL-2 is pre-formed in the cytoplasm of LBRM-33 and is released into the medium after mitogen sensitization. The observation that increasing concentrations of IL-2 are found over a 16 hr time period in supernatants of stimulated LBRM-33 cells (see Fig. 1) also argues against the hypothesis that mitogen activation simply stimulates cell death and the concomitant release of pre-formed IL-2. Another possibility of course is that the IL-2 produced by LBRM-33 cells is somehow cytotoxic to them. In such a system,

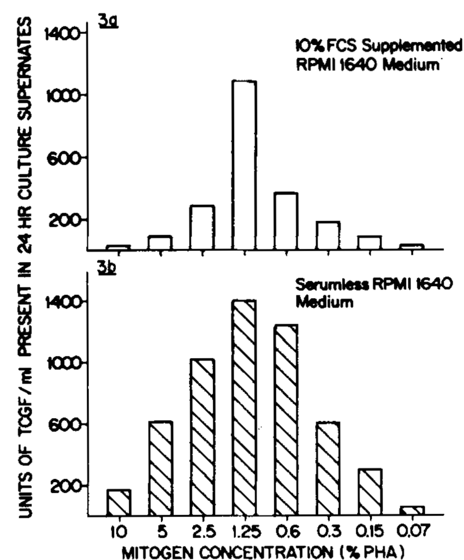

Figure 3. Twenty-four hr, LBRM-33 IL-2 production after culture (10^6 cells/ml) with varying concentrations of PHA in either FCS-supplemented (*A*) or serum-free (*B*) RPMI 1640.

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IL-2 production would therefore be self-governing. However, experiments in which murine (6), rat, or human (7) IL-2 were added to LBRM-33 cells failed to show any appreciable difference in cellular metabolism or viability (data not shown).

Use of phorbol myristate acetate (PMA) to enhance IL-2 production of LBRM-33 cells at suboptimal mitogen doses. Several groups (19, 21) have recently found that the addition of the monokine Interleukin 1 (IL-1 previously referred to as lymphocyte activating factor or LAF) to adherent cell-depleted murine T cells restores the capacity of such cells to produce IL-2 after mitogen sensitization. Based on the results of Farrar and Fuller-Bonar (22) who found that the unsaturated fatty acid derivative, phorbol myristate acetate (PMA), provided a similar macrophage replacement for T cell IL-2 production and furthermore acted to boost IL-2 produced by mixed T cell/macrophage cultures, we examined the effects of the addition of PMA to mitogen-stimulated LBRM-33 cultures. PMA (10 to 50 ng/ml) was added either alone or together with mitogen at the initiation of microplate LBRM-33 cultures. Replicate cultures were conducted that contained either optimal or suboptimal concentrations of Con A or PHA (Fig. 3, 4). As detailed in Table II, addition of PMA to LBRM-33 cultures co-stimulated with optimal concentrations of mitogen resulted in no increase in 24 hr IL-2 supernatant titer. Stimulation with PMA alone resulted in no IL-2 production. However, addition of PMA to LBRM-33 cells co-cultured with a suboptimal mitogen concentration (either Con A or PHA) increased IL-2 production in such cultures to a level equal to that generated by cells stimulated with optimal amounts of mitogen. Unfortunately, PMA treatment of LBRM-33 cells together with suboptimal amounts of Con A or PHA resulted in LBRM-33 cell death just as was observed in cultures stimulated with peak mitogen concentration (Fig. 4). Therefore the results detailed in Table II provide further evidence in support of the close correlation between peak IL-2 production and concomitant LBRM-33 cell death.

Search for pharmacologic agents that might induce LBRM-33 IL-2 production. Although mitogen-stimulated LBRM-33 cells generated greater than 1000 times the amount of IL-2 normally obtained from conventional mouse or rat splenotype cultures, we were curious to determine whether a lower m.w. metabolic perturbant might be capable of provoking LBRM-33 lymphoma cells to produce IL-2 in the absence of mitogen. The

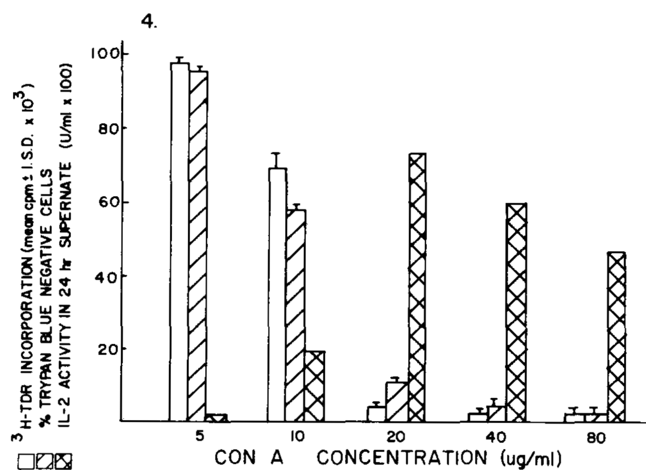

Figure 4. Relationship of IL-2 production and LBRM-33 cell viability. Supernatant IL-2 titers (▧), culture viability (▨), and LBRM-33 cellular proliferation (□) are compared after stimulation with varying concentrations of Con-A.

TABLE II

Effect of phorbol myristate acetate (PMA) on LBRM-33 IL-2 production

LBRM Culture Stimulants			% Viable LBRM-33 Cells Remaining after 24-h Culture	IL-2 Activity Present in 24-h Culture Supernatant
PHA	CON A	PMA		
				U/ml
—[1]	—	—	100	0.0
—	—	10 ng/ml	95	0.0
—	—	30 ng/ml	98	0.0
—	—	50 ng/ml	100	0.0
—	20 μg/ml	—	4	783.0
—	20 μg/ml	10 ng/ml	3	815.0
—	20 μg/ml	30 ng/ml	3	762.0
—	20 μg/ml	50 ng/ml	6	801.0
—	2 μg/ml	—	87	16.0
—	2 μg/ml	10 ng/ml	13	673.0
—	2 μg/ml	30 ng/ml	4	712.0
—	2 μg/ml	50 ng/ml	3	706.0
1%	—	—	2	864.0
1%	—	10 ng/ml	1	912.0
1%	—	30 ng/ml	6	831.0
1%	—	50 ng/ml	5	869.0
0.1%	—	—	91	26.0
0.1%	—	10 ng/ml	6	935.0
0.1%	—	30 ng/ml	5	932.0
0.1%	—	50 ng/ml	3	869.0

[1] —, Absent from culture.

benefit of such a stimulant would be in its capacity to be removed from serum-free medium via simple dialysis as opposed to the present 4 to 5 step purification required for purification of mitogen free murine IL-2 (6). As detailed in Table III, we tested several reagents for their ability to induce IL-2 LBRM-33 production in replicate 24 hr microwell cultures. We tested several flux inducers of cytoplasmic cyclic nucleotide concentration due to the well established role of these compounds in metabolism and secretion (10). Thymopoietin and its active pentapeptide TP5 were tested at concentrations previously shown by other investigators to have marked effects on T cell differentiation and function (10, 11). As detailed in Table III, no compound tested either at pharmacologic or physiologic concentration was capable of eliciting IL-2 production that in any way approached quantities produced upon stimulation with mitogen. The sole compound that gave any response was carbachol that when used to stimulate LBRM-33 cells at a concentration of 10^{-3} M generated 10.0 U/ml of TCGF in a 24 hr supernatant. The capacity of carbachol to produce such a small amount of IL-2 production most likely could not be traced to its ability to increase cytosol concentrations of cyclic GMP in that other reagents that produce a similar effect (dibutyryl cyclic GMP) were incapable of evoking IL-2 production.

Phenotypic characterization of LBRM-33. Wagner and Rollinghoff (23) recently reported that pretreatment of murine spleen cells with anti Ly 1 serum and C abolished the capacity of that population to produce IL-2 in response to mitogen/antigen stimulation. This has led to the general belief that the IL-2 producer cell is on Ly 1+ T cell. To further characterize LBRM-33 as an IL-2 producer, we assayed the cell line for the surface expression of a number of phenotypic markers in a direct, antibody-dependent, protein A-coated SRBC-rosetting

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assay. Eight separate trials were conducted with sera specific for Thy 1, TL antigen, Ly 1, Ly 2, Qa 2–3, Qa 3, Qat 4, and Ly 5 markers. As shown in Table IV (in terms of the number of rosettes found per 100 cells observed after treatment with a given serum) LBRM-33 cells were highly Thy 1 and Ly 5 positive. The cell line showed significant reactivity with all other typing sera, in particular the anti-TL, Qat 4 reagents.

The LBRM-33 cell surface expression of Ly 1.2 and Ly 3.2 (2.2) was confirmed in quantitative absorption tests. LBRM-33 cells were tested at 3 different cell concentrations (5×10^7, 10^8, and 2×10^8 cells/ml) for their capacity to remove the cytolytic reactivity from a 1/200 dilution of either anti-Ly 3.2 or anti-Ly 1.2 sera. Absorbed sera were then tested for their ability to lyse normal C57BL/6 thymocytes in antibody dependent, C-mediated cytolysis assays. As shown in Figure 5*A*, LBRM-33 absorbed anti-Ly 3.2 sera reactivity in a cell concentration dependent manner (50% absorption at a concentration of 2×10^8 cells/ml) whereas thymocytes harvested from the B6 Ly 3.1 congenic mouse were incapable of absorbing lytic reactivity. Similarly, LBRM-33 removed 50% of the lytic activity of a 1/200 dilution of anti-Ly 1.2 sera in quantitative absorptions (2×10^8 cells/ml) whereas B6 Ly 1.1 thymocytes absorbed no cytolytic reactivity. Although LBRM cells were capable of absorbing anti-Ly 1.2 and anti-Ly 3.2 reactivity, it is likely that the surface density of either of these markers is relatively low, in that normal C57BL/6 thymocytes were far more effective in absorption trials (Fig. 5*A* and 5*B*) despite the observation that resting thymocytes are essentially one-third to one-fourth the size of an LBRM-33 cell. We are confident however that LBRM-33 cells do express Ly 1 and Ly 3 (Ly 2) markers, since neither these cells nor B6 Ly 1.2 thymocytes (10^8 cells/ml) demonstrated any ability to absorb cytolytic reactivity from an anti Ly 1.1 serum as tested on C56BL/6 Ly 1.1 thymocytes (Fig. 5*C*).

TABLE III

Compounds tested for induction of LBRM-33 IL-2 production

Compound	Concentration	IL-2 Activity in 24-hr Culture Supernatant
		U/ml
PHA	0.5%	406.0
	1.0%	1100.0
	2.0%	875.0
CON A	2 μg/ml	100.0
	10 μg/ml	300.0
	20 μg/ml	800.0
8-Bromo cyclic AMP	10^{-5} M	0.0
	10^{-6} M	0.0
	10^{-7} M	0.0
Di-butyryl cyclic AMP	10^{-3} M	0.0
	10^{-4} M	0.0
	10^{-5} M	0.0
	10^{-6} M	0.0
	10^{-7} M	0.0
Di-butyryl cyclic GMP	10^{-3} M	0.0
	10^{-4} M	0.0
	10^{-5} M	0.0
	10^{-6} M	0.0
	10^{-7} M	0.0
Carbachol	10^{-3} M	10.8
	10^{-4} M	0.8
	10^{-5} M	0.0
	10^{-6} M	0.0
Isoproterenol	10^{-3} M	0.0
	10^{-4} M	0.0
	10^{-5} M	0.0
Indomethacin	10^{-4} M	0.0
	10^{-5} M	0.0
	10^{-6} M	0.0
Prostaglandin E_1	10^{-4} M	0.0
	10^{-5} M	0.0
	10^{-6} M	0.0
Prostaglandin E_2	10^{-4} M	0.0
	10^{-5} M	0.0
	10^{-6} M	0.0
Thymopoietin	10 μg/ml	0.0
	5 μg/ml	0.0
	2 μg/ml	0.0
TP5	100 μg/ml	0.0
	10 μg/ml	0.0
	1 μg/ml	0.0

DISCUSSION

Although it is questionable whether analysis of a functional malignant cell is directly applicable to the normal condition, study of immunologically reactive myelomas has led in recent years to significant understanding of the triggering and function of normal antigen reactive B lymphocytes (24, 25). Similarly, application of studies of tumor cell lines has allowed for a rapid advancement in the understanding of the molecular characteristics and functional reactivity of several lymphokine/monokine activities most notably colony-stimulating factor (26) and Interleukin 1 (27) (formerly referred to as LAF). Therefore the isolation and characterization of the extremely efficient IL-2 producer lymphoma, LBRM-33, provides several new insights into the possible origin and regulation of IL-2 production.

Based primarily on the phenotypic analysis of LBRM-33 (Fig. 5 and Table IV) and to a lesser extent on the capacity of the cell line to produce IL-2 only in response to T cell mitogens (Table I), it appears that the cellular origin of IL-2 is a T lymphocyte. The observation that LBRM-33 cells clearly express all three Lyt antigens at the cell surface should not be construed as contradictory to the studies of Wagner and Rollinghoff (23) who found that elimination of Lyt 1 antigen positive splenocytes totally abrogated antigen driven IL-2 production. It is conceivable that the phenotyping methods described in this report (protein A-SRBC rosetting and quantitative absorption) are better suited for detecting low density Ly antigen expression than are direct C-dependent cytotoxicity regimens. Alternatively, it is conceivable that the LBRM-33 cell line represents a cell transformed after acquisition of IL-2 production capacity yet prior to cell surface evolution to a predominant Lyt 1 surface phenotype, or for that matter loss of the characteristic immature T cell surface marker, the TL antigen.

It is clear from the data presented in this report that LBRM-33 cells are capable of producing extremely large amounts of IL-2 in the absence of an accessory cell population. Several groups have previously reported that adherent cell-depleted normal T cell populations are extremely deficient in their capacity to produce IL-2 (19, 21). However, normal levels of IL-

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TABLE IV

Phenotypic characterization of LBRM-33 cells in direct protein A sheep erythrocyte rosette assay

Cell Line	Number Trials	% Rosette Positive LBRM-33 Cells following Treatment with Protein A-coated SRBC and Antiserum Directed against							
		Thy +	TL	Ly 1	Ly 2	Qa 2-3	Qa 3	Qa T4	Ly 5
LBRM-33	8	95.0 ± 16	41 ± 6	35 ± 8	37 ± 6	30 ± 4	33 ± 6	38 ± 7	85 ± 7
C57BL/6 splenocytes	5	39.4 ± 4	N.T.[1]	40.2 ± 2.4	19.2 ± 2	78.6 ± 7.0	N.T.	44 ± 5.2	89 ± 5.2

[1] N.T., not tested.

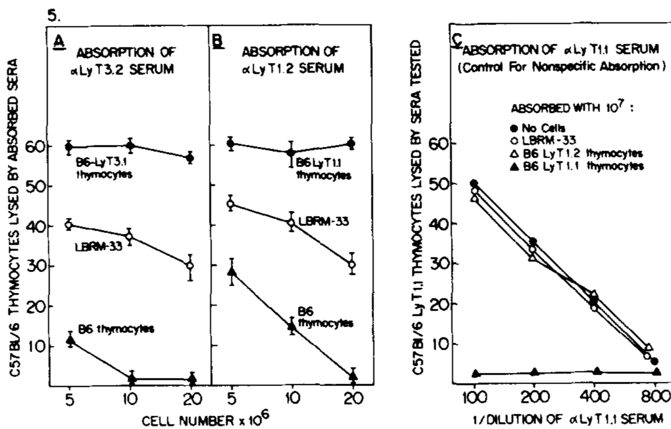

Figure 5. Confirmation of LBRM-33 Ly T phenotype by absorption. *A*, absorption of α Ly T 3.2 serum cytotoxic reactivity (1/200 dilution) by C57BL/6 Ly T 3.1 thymocytes (●), LBRM-33 (○), and C57BL/6 Ly T 3.2 thymocytes (▲). *B*, absorption of α Ly T 1.2 serum cytotoxic reactivity (1/200 dilution) by C57BL/6 Ly T 1.1 thymocytes (●), LBRM-33 (○), and C57BL/6 Ly T 1.2 thymocytes (▲). *C*, absorption of α Ly T 1.1 cytotoxic reactivity by C57BL/6 Lyt T 1.2 thymocytes (△), C57BL/6 Ly T 1.1 thymocytes (▲), LBRM-33 (○), and in the absence of any cell population (●).

2 generation can be restored by reconstitution with 0.5% adherent monocytes or monocyte condition medium (19). Most recently, the entity present in such conditioned media (capable of restoring purified T cell IL-2 production) has been identified as Interleukin 1 (LAF, 21). Such experiments have led to the belief that a key functional characteristic of the IL-1 molecule is to trigger reactive T cells to become IL-2 producers. Whether the interaction of IL-1 with a particular T cell subpopulation represents a growth factor cascade effect wherein IL-1 directly evokes IL-2 production has not been addressed. Alternatively, it is conceivable that current reconstitution data could be explained due to a differentiation-induction effect of IL-1 and the conversion of a non-IL-2 producer cell into a producer population. It is hoped that use of LBRM-33 cells (both producer and nonproducer subclones) together with purified IL-1 might provide suitable reagents for dissecting the relationships between IL-1 activity and IL-2 production. In this regard, it is interesting to note that although LBRM-33 cells clearly do not need accessory cell products to produce IL-2 after optimal mitogen stimulation (Table I), data detailed in Table II showed that the same cells were capable of responding to a substitute for IL-1 (PMA) to produce large amounts of IL-2 in cases of suboptimal mitogen stimulation.

One of the most interesting facets of LBRM-33 IL-2 production is the consistent observation (Table II, Fig. 4) that IL-2 production is accompanied by cell death. The most appealing aspect of such a reaction is its potential applicability to the normal condition. There has been no evidence presented to date to support the hypothesis that after ligand activation, the IL-2 producer T cell either i) responds to IL-2 and proliferates or ii) merely survives. If IL-2 producer cells were capable of responding to IL-2, one would expect that after mitogen/antigen sensitization, such cells would continue proliferating by their own accord, if not indefinitely, at least to the degree where they would represent a substantial portion of the cells surviving after either mitogen stimulation or MLC activation. Clearly this is not the case. Factor responsive cells have been grown indefinitely in culture only by the continual addition of IL-2 (1-7). In all cases such cells lines have been shown to mediate effector T cell functions (to date helper or killer) and have not been found to demonstrate capacity for IL-2 production (Table I).

Furthermore, it is conceivable that an IL-2 producer cell that was capable of responding to its own IL-2 production would represent a self-perpetuating (autokine) stimulation that in its simplest example might represent leukemic proliferation. Such autostimulatory roles for factor production and use have recently been invoked in the relationship of sarcoma growth factor and the sustained proliferation of sarcoma virus-transformed cells (28). Similar repetitive antigen stimulation has also been suggested as a potential explanation for the proliferation observed in virus-induced T cell lymphomas (29). Given the knowledge that IL-2 is responsible for antigen-stimulated T cell proliferation, the involvement of IL-2 in the unchecked replication of potentially antigen-driven leukemic T cells may be considerable.

In addition to serving as a useful reproducible reagent for studying the effects of pharmacologic reagents on IL-2 production (Table III), it is hoped that LBRM-33 cells will be a valuable source for the preparation of internally radiolabeled IL-2. The inability to isolate IL-2 activity from sonicates of LBRM-33 cells supports the hypothesis that IL-2 is not preformed in the cytoplasm. However, it will be necessary to study the effects of protein and DNA synthesis inhibitors on LBRM-33 IL-2 production to determine the best possible protocol for internal labeling as well as to further dissect the relationship of cell death and IL-2 production. At the very least, the use of a cell line that produces between 1000 and 5000 times the amount of IL-2 activity generated from conventional sources should greatly aid in the purification and further molecular characterization of this lymphocyte regulatory molecule. The following manuscript addresses the molecular characteristics of LBRM-33-derived IL-2 and compares its activity and properties to that of conventionally generated murine IL-2 prepared from mitogen-activated spleen cells.

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