GENERATION OF CONTINUOUS LARGE GRANULAR LYMPHOCYTE LINES BY INTERLEUKIN 2 FROM THE SPLEEN CELLS OF MICE INFECTED WITH MOLONEY LEUKEMIA VIRUS

Involvement of Interleukin 3

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Large granular lymphocytes (LGL)[1] represent a morphologically defined, distinct population of lymphoid cells, although they do not necessarily seem to originate from a single lineage of population in the lymphohematopoietic system (1, 2). The major functions of LGL so far defined include MHC-unrestricted cytotoxicity, so-called NK activity, and negative regulation of hematopoiesis (3, 4). They are under the physiological regulation of a number of cytokines, which mediate the responsiveness of LGL in vivo to a variety of external stimuli. IFNs, for instance, potentiate the cytotoxic activity of LGL (5), whereas IL-2 primarily acts as a growth factor (6). In normal blood and lymphoid tissues in both human and mice, LGL seem to be a major population to initiate proliferation by the sole stimulation with IL-2 in the absence of any particular antigenic stimulation (7, 8). The proliferative potential, however, is generally limited, and sooner or later LGL lose the proliferative capacity even in the continuous presence of IL-2 in vitro. The continuous IL-2-dependent LGL lines could be infrequently established from spleen cells of mice primed in vivo with NK-susceptible cells, such as certain leukemia cells and conventional virus-infected cells, only in the simultaneous presence of macrophages (Mϕ) or activated Mϕ-derived factor (IL-1) in addition to rIL-2 (9). It was indicated that one of the major roles of Mϕ was to maintain high affinity IL-2-R expression on LGL, suggesting that the restricted proliferative capacity was partly due to the progressive loss of functional IL-2-R expression (9). Meanwhile, we have found that continuous LGL lines could be easily established with nearly 100% efficiency by IL-2 alone in the

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[1] *Abbreviations used in this paper:* Ab-MLV, Abelson murine leukemia virus; GM-CSF, granulocyte/macrophage colony-stimulating factor; Ki-MSV, Kirsten murine sarcoma virus; LGL, large granular lymphocytes; Mo-MLV, Moloney murine leukemia virus; Mϕ, macrophage; RT, reverse transcriptase.

absence of particular feeder cells from the spleen cells of mice injected with Moloney leukemia virus (Mo-MLV)-producing cell lines or infected directly with Mo-MLV. Based on this observation, the present report reveals the conditions and molecular requirements for the unlimited IL-2-dependent proliferation of LGL. The implications of the mechanism in the differentiation of LGL, as well as the regulation of hematopoiesis and leukemogenesis, will be discussed.

Materials and Methods

Mice. BALB/c (+/+ and *nu/nu*), DBA/2, and C57BL/6 mice were purchased from Shizuoka Experimental Animal Center, Schizuoka, Japan. C57BL/6-*bg/bg* (beige) mice were bred in our own colonies. CB17 (+/+ and *scid/scid*) mice were kindly provided by Dr. Taniguchi (Kanazawa University, Kanazawa, Japan) from the original breeding pairs of Dr. Bosma, The Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA. (10).

Cell Lines and Viruses. Murine cell lines, YAC-1, RLδ1, P815, and EL4 were all maintained in RPMI 1640 medium supplemented with 10% FCS, 5×10^{-5} M 2-ME, 50 U/ml penicillin, and 50 μg/ml streptomycin (10% FCS-RPMI). A murine IL-2-dependent CTL line, CTLL-2, was maintained in 10% FCS-RPMI supplemented with human rIL-2. IL-3-dependent cell line, FDC-P2, was cultured in 10% FCS-RPMI containing 25% WEHI-3-conditioned medium.

Retrovirus-producing cell lines used for these studies were as follows: a producer clone of Kirsten murine sarcoma virus (Ki-MSV)-transformed NIH/3T3 cells (12NY-C165) was kindly provided by Dr. Y. Ikawa of the Institute of Physical and Chemical Research, Tsukuba, Japan; Rat-1 Mo-MLV producer (MIT/MLV) (11) and Rat-2 (tk^-) lines were kindly provided by Dr. M. Krigler of the University of California, Berkeley, CA; a producer clone of Abelson leukemia virus (Ab-MLV)-transformed NIH/3T3 (tk^-) cells (Ab.NIH-c12) was a kind gift from Dr. M. Noda of the Institute of Physical and Chemical Research. These cell lines were all cultured in Dulbecco's modified MEM containing 10% FCS and antibiotics.

Virus stocks for infection of mice were clarified and concentrated culture fluids from producer cell lines. The titer of replication-competent Mo-MLV was determined by the UV-XC plaque assay (12), and the titer of transforming Ab-MLV and Ki-MSV was determined by focus formation on Rat-2 cells (13).

Establishment of IL-2-dependent LGL Lines. IL-2-dependent continuous LGL lines were established using retroviruses as follows. Mice were subcutaneously injected with various kinds of retroviruses or their producer lines. After a few weeks, the spleen cells obtained from each mouse were cultured at 10^6 cells/ml in 10% FCS-RPMI in the presence of rIL-2 (100 U/ml) alone without any additive feeder cells in a T-25 flask (Corning Glass Works, Corning, NY). In a few weeks, the growing cells became apparent and such cells could be successfully expanded in the continuous presence of rIL-2 alone.

Phenotypical Analysis. Surface phenotypes of the LGL lines were analyzed using a FACS 420 (Becton Dickinson & Co., Mountain View, CA). Antibodies used included monoclonal anti-Thy-1.2 (IgM), anti-Lyt-1.2 (IgG2b), anti-Lyt-2.2 (IgM), anti-L3T4 (GK 1.5), rabbit anti-asialo GM_1 ($AsGM_1$:IgG fraction), and monoclonal anti–mouse IL-2-R antibody (AMT13). Fc receptor for IgG (FcR) was examined by rosette-forming assay using sheep erythrocytes sensitized with rabbit anti–sheep erythrocyte antibodies.

Cytotoxicity Assay. Cytotoxic activity of the lines was assayed using the ^{51}Cr-release method as described before (9). Assay time was 5 h for lymphoma and normal bone marrow cell (BMC) targets and 8 h for adherent cells. Calculation of specific ^{51}Cr-release was described before (1).

Cell Freezing. The established cell lines were frozen at various stages using a computer-programmed cell freezer (model 801; Cryo-Med, Mt. Clemens, MI). Frozen cells could be successfully thawed in the initial presence of irradiated peritoneal adherent cells.

IL-2, IL-3, and Their Assay. The genes for human IL-2 and murine IL-3 were cloned from the cDNA libraries of tonsillar mononuclear cells and the 1H5.5 murine cell line

(14), respectively, using the synthesized oligonucleotides based on the published sequences. Each of them was expressed in *Escherichia coli* and COS-1 cells using pkM6 (15) and pcD-X (16) vector, respectively. Each activity was assayed using CTLL-2 and FDC-P2 cell lines as an indicator, respectively, by 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyl petazoium bromide) (MTT) colorimetric assay system (17). One unit represented a dilution factor that provided 50% of the maximum proliferation of corresponding indicator cells, and the titers of rIL-2 and IL-3 were determined by dose-response titration curves. rIL-2 was chromatographically purified from *E. coli* extracts, specific activity being 10^7 U/mg protein. As rIL-3, serum-free culture supernatant of transfected COS-1 cells was used, which had a specific activity of 10^5 U/mg protein. Some IL-3 dependent cell lines were reported to respond not only to rIL-3 but to recombinant granulocyte/macrophage colony-stimulating factor (rGM-CSF) but the FDC-P2 line in our hands did not respond to rGM-CSF at all. In some experiments, WEHI-3-conditioned medium, which contained 100 U/ml of IL-3 activity without detectable IL-2 activity, was also used.

Cellular Binding Assay with ^{125}I IL-2. Cellular ^{125}I-labeled IL-2 binding assay was performed as described before (9). Briefly, target cells were incubated in 10% FCS-RPMI containing radio-labeled rIL-2. After 20 min incubation, the cells were washed with fresh medium and were centrifuged on a layer of the mixture of dibutylic acid and olive oil. The free as well as cell-bound radioactivity were counted with a gamma counter. The specific binding of IL-2 was calculated by subtracting the background radioactivity that was not competed by 500-fold excess of cold rIL-2.

Interferon Assay. IFN activity was assayed using the microcytotoxicity inhibition test as described before (1). Briefly, dilutions of samples were incubated with monolayers of L929 cells in microtiter wells for 24 h. After draining the samples, L929 cells were challenged with encephalo-myocarditis virus (EMCV), and 24 h later the cytopathic effects were checked by Multiscan (Flow Laboratories, Inc., McLean, VA) after vital staining with crystal violet. 1 U represented a dilution factor that provided 50% protection of cytopathic effect by EMCV.

Southern Blot Hybridization. DNA from BALB/c liver cells and LGL lines were prepared using standard methods (18). To examine whether Mo-MLV was integrated into the DNA of cell lines or not, DNA (10 μg each) was digested with either Bam HI or Eco RI, electrophoresed on 0.8% agarose, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled Mo-MLV Sma I fragment as a probe. The probe specific for the *env* region of Mo-MLV was purified from Mo-MLV cDNA clone (19), kindly provided by Dr. O. Niwa of Hiroshima University, Hiroshima, Japan. The filters were hybridized overnight and washed at 65°C with 0.1× SSC plus 0.1% SDS. To analyze the TCR genes of LGL lines, 10 μg of DNA of each sample was digested with either Pvu II or Hind III, and hybridized with a ^{32}P-labeled TCR β chain gene probe. The probe specific for the constant region gene of TCR-β chain (86T5) was kindly provided by Dr. M. M. Davis of Stanford University, Stanford, CA. The filters were hybridized and washed as previously described (20).

Reverse Transcriptase (RT) Assay. RT assay was performed as described by Goff et al. (21). Briefly, subconfluent cells were fed with fresh medium 12 h before being assayed to maximize virus production. 30–40 ml of culture fluids were filtered through a 0.22-μm millipore filter and concentrated by ultracentrifuging for 60 min at 8,000 *g*. Then the pellet was resuspended in 20 μl of TNE buffer (0.1 M NaCl, 0.01 M Tris-HCl, 0.1 mM EDTA, pH 7.2) and referred to as an RT sample.

Results

Reproducible Generation of Continuous Cell Lines In Vitro by IL-2 Alone from the Spleen Cells of Mice Infected with Mo-MLV. Normal BALB/c mice were injected subcutaneously with 12NYC165, a NIH 3T3 cell line transformed with Ki-MSV, and 2–3 wk later the spleen cells were cultured with 100 U/ml of rIL-2 without any additive stimulation. Cultures were fed with rIL-2 every other day. Within 2–3 wk, cell proliferation became apparent in the cultures from every single

TABLE I

Generation of Continuous Cell Lines by rIL-2 alone from the Spleen Cell Cultures of Mice Inoculated with Retroviruses or Their Producer Cell Line

Exp.	Inocula	Cell number or virus titer	Strains of mice	Cell lines per number of mice
A	None		BALB/c, +/+	0/10
	12NY-C165 cells	10^6 cells	BALB/c, +/+	8/12
B	Ki-MSV/Mo-MLV	10^5 PFU	BALB/c, +/+	3/3
	Ab-MLV/Mo-MLV	10^6 PFU		3/3
	Mo-MLV	10^7 FFU		5/5
C	12NY-C165 cells	10^6 cells	DBA/2, +/+	2/2
			C57BL/6, +/+	2/2
			CB-17, +/+	2/2
			BALB/c, *nu/nu*	0/13
			C57BL/6, *bg/bg*	3/3
			CB-17, *scid/scid*	0/4

Mice were inoculated subcutaneously with 12NY-C165 cells, retrovirus-producer cells, or various retrovirus preparations. 2 wk later, the spleens were cultured in the presence of rIL-2 as described in Materials and Methods. The establishment of cell lines was judged when the growing cells could be propagated at least several passages in the presence of rIL-2. Some of them were maintained over a year with continuous IL-2-dependent proliferation.

mouse. Such cells could now be continuously propagated in the presence of rIL-2, resulting in the cell lines (Table I, Exp. A). Initial cell proliferation was observed also in the spleen cell cultures from normal mice by rIL-2, but the cultures died off regularly within 2 wk even in the presence of rIL-2, and propagatable cell lines could hardly be obtained (Table I, Exp. A). Since 12NYC165 cells produced abundant retroviruses as described in Materials and Methods, mice were injected with the ultracentrifuged virus preparations from various retrovirus-producer cell lines (Ki-MSV/Mo-MLV, Abl-MLV/Mo-MLV), and the spleen cells were cultured with rIL-2 with the same results (Table I, Exp. B). The producer cell lines commonly possessed Mo-MLV as helper virus, and thus we infected mice with purified Mo-MLV again with the same observation (Table I, Exp. B). It thus seemed that Mo-MLV infection alone was sufficient for in vivo conditioning of mice for the subsequent generation of continuous cell lines in vitro by the sole stimulation with rIL-2, although it remains to be seen whether other retroviruses can do the same.

IL-2-dependent continuous cell lines were also generated from other strains of mice by retroviruses (Table I, Exp. C). In contrast, no such cell lines were ever generated from the spleen cells of BALB/c nude and CB-17 scid homozygote mice injected with 12NYC165 cells, while the lines developed from heterozygote mice of both strains (Table I, Exp. C). The results indicate that functional T cells are in some way required for the IL-2-induced generation of cell lines after Mo-MLV infection. In beige mice, which have intact T cells but a functional defect in the NK cell population, the cell lines could be successfully developed.

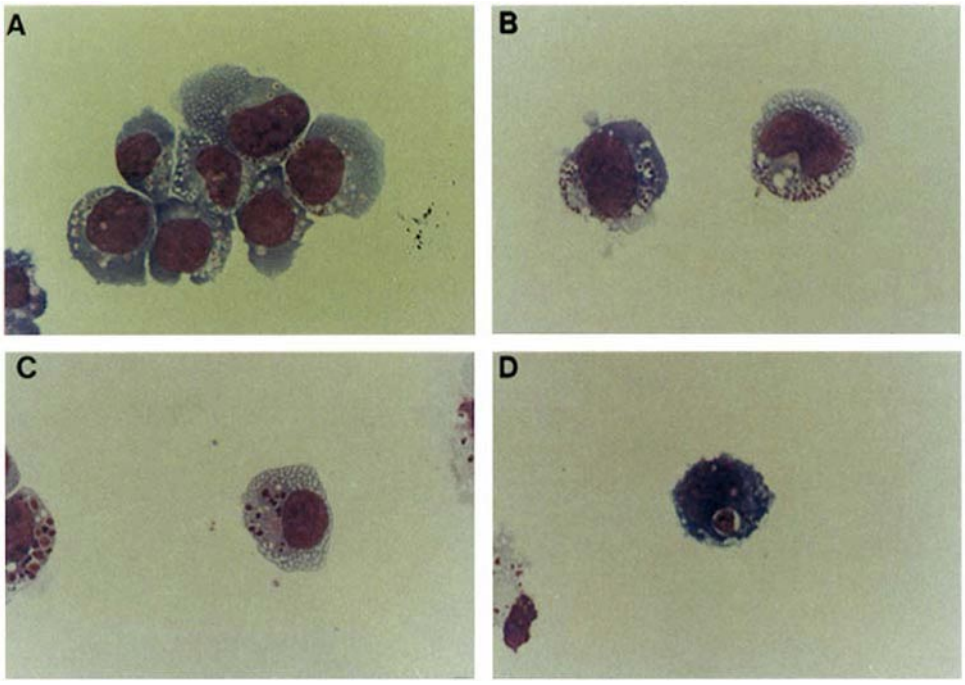

FIGURE 1. Morphology of continuous cell lines generated from the spleen cells of various strains of mice infected with Mo-MLV (May-Giemsa stain, × 1,500). (*A*), BALB/c, (*B*) C57BL/6, (*C*) DBA/2, (*D*) C57BL/6-*bg*/*bg*.

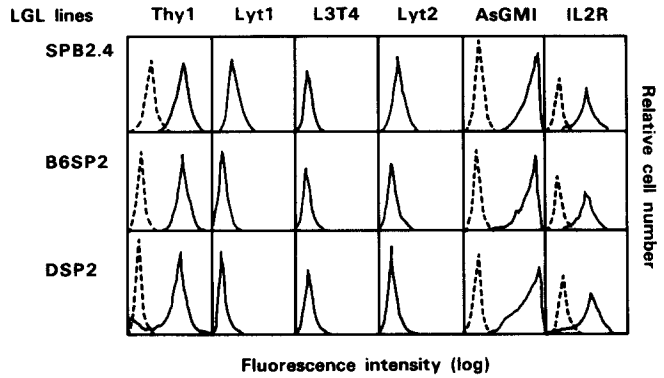

FIGURE 2. Surface phenotype analysis of LGL lines by flow cytometry. Dotted lines indicate negative controls by the FITC-conjugated second antibody alone.

Characterization of the Cell Lines as LGL with NK Activity. As shown in Fig. 1, *A–C*, all of the cell lines showed typical morphological features as LGL, without exception. The lines derived from MLV-infected beige mouse spleen cells had aberrant gigantic granules in the cytoplasms (Fig. 1 *D*), reflecting genetic anomalies in the granulated cells in this mutant strain. It should be noted that no preselective procedures were performed before setting up the cultures. Nevertheless, the established lines were quite homogeneous in terms of morphology. The results of phenotypical analysis are summarized in Fig. 2. Again, the

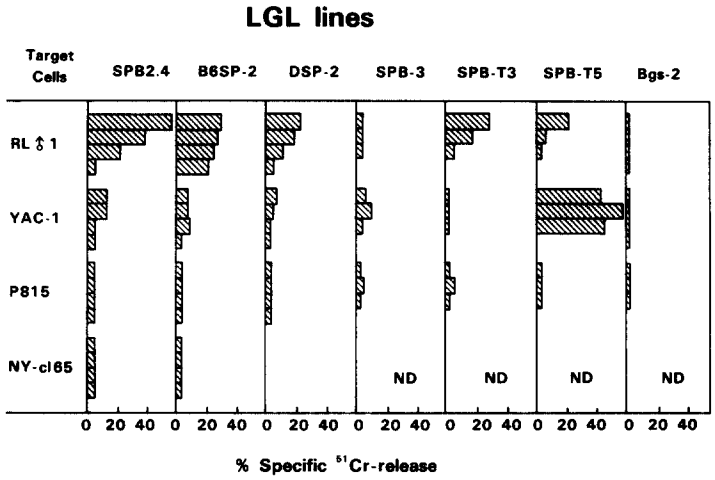

FIGURE 3. Cytotoxic activity of LGL lines. Percent specific ^{51}Cr-release at 10:1, 3:1, 1:1, and 0.3:1 E/T ratios, respectively from the top bar, are shown.

TABLE II

Reverse Transcriptase Activity of Culture Supernatants of LGL Lines Generated from the Spleen Cells of Mice Infected with Mo-MLV

Cell lines	RT activity
	cpm
Buffer	252
Normal spleen cells	430
Mo-MLV infected spleen cells*	55,490
Producer cell lines	
MIT/MLV	610,672
12NY-C165	948,321
LGL lines	
SPB 2.4	581
SPB 3	412
SPB-T3	136
BSP-2	501
DSP-2	1,159
SPB-VI	1,549

* Spleen cells from Mo-MLV-infected mice were cultured for 4 d by the same conditions to develop LGL lines (Materials and Methods). The culture supernatants were harvested and concentrated for RT assay.

serological phenotype was quite uniform, and all the lines showed the same phenotype, namely Thy-1$^+$,Lyt-1$^-$,L3T4$^-$,Lyt-2$^-$, AsGM$_1^+$,IL-2-R$^+$,FcRγ^+. The morphological and phenotypical characteristics of these lines represented those of a set of NK cells in the spleen, and thus the cytotoxic activity was then examined. As shown in Fig. 3, most of the lines showed various degrees of NK-patterned cytotoxic activity. On the other hand, they did not affect 12NYC165 cells at all, indicating that the cytotoxicity was not specifically directed to Mo-MLV used for the in vivo stimulation. LGL lines from beige mice (Bgs-2) failed to show any cytotoxic activity against NK-susceptible RL♂1 and YAC1-cells.

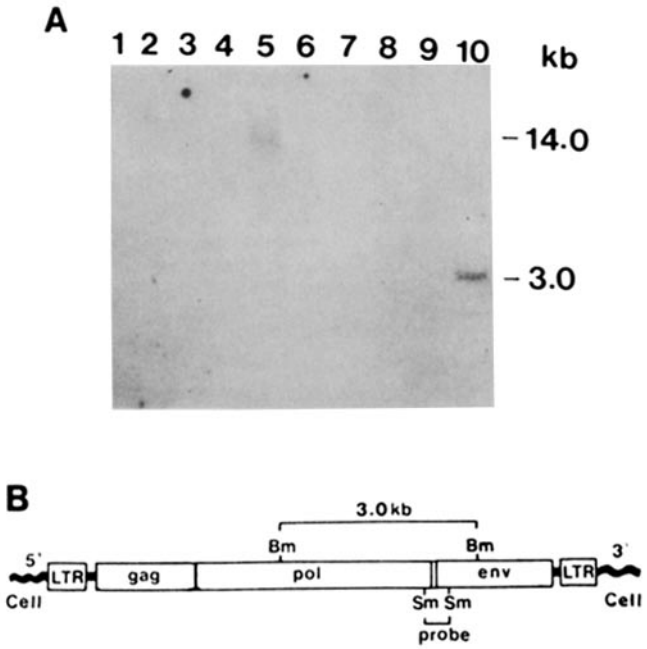

FIGURE 4. Southern blot analysis of the DNA from LGL lines using a Sma I (*Sn*) fragment of Mo-MLV genome. DNA from three different LGL lines developed from Mo-MLV-infected mice were digested with either Eco RI (lanes *1–3*) or Bam HI (*Bm*) (lanes *6–8*). DNA from BALB/3T3 clone A-31 (lanes *4* and *9*) and MIT/MLV (lanes *5* and *10*) were similarly treated as a negative and positive control, respectively. 10 μg of each DNA was then electrophoresed, transferred to a filter, and hybridized with a ^{32}P-labeled Mo-MLV probe (see the diagram), as described in Materials and Methods. 3.0-kb *Bm* hybridization fragment of Mo-MLV with the probe is also illustrated.

LGL Lines from MLV-infected Mice Had No Infectious MLV nor Proviral Integration. To understand the mechanisms for the conditioning of LGL in vivo by Mo-MLV infection, the first question to be addressed was whether MLV infected the lines. For this, RT activity of the culture supernatants of LGL lines was first examined. As shown in Table II, no significant RT activity was detected even with the concentrated LGL culture supernatants, whereas culture supernatant of total splenic cells from Mo-MLV-infected mice showed significant RT activity. Producer cell lines exhibited high RT activity. We then analyzed the possible Mo-MLV proviral integration in these LGL lines. Bam HI– or Eco RI–digested DNA of the LGL lines or MLV-transformed line was electrophoresed, transferred onto nitrocellulose filters, and hybridized with ^{32}P-labeled Sma I fragment of the cloned Mo-MLV gene, which is specific for the *env* region of exogenous Mo-MLV. As shown in Fig. 4 (lane *10*), the 3.0-kb hybridization band was clearly detected in the Bam HI–digested DNA from Rat-2 line persistently infected with Mo-MLV (MIT/MLV), which represented the *pol-env* region of exogenous Mo-MLV proviral genome. In contrast, no 3.0-kb band was detected at all in any of LGL lines derived from Mo-MLV-infected mice (Fig. 4, lanes *6–9*).

Taken together, these results indicated that Mo-MLV was not directly infected in the LGL lines.

Mo-MLV Infection does not Affect Splenic NK Activity per se but Induces IL-3 Production In Vivo. We then inquired the alternative possibility that MLV affected LGL indirectly by inducing certain factors in the infected hosts. IFNs and IL-2 are well-known factors that affect the activity and/or proliferation of LGL-NK cells in vivo, and a number of conventional viruses, viral-infected cells, or tumor cells are shown to influence NK activity through these factors. However,

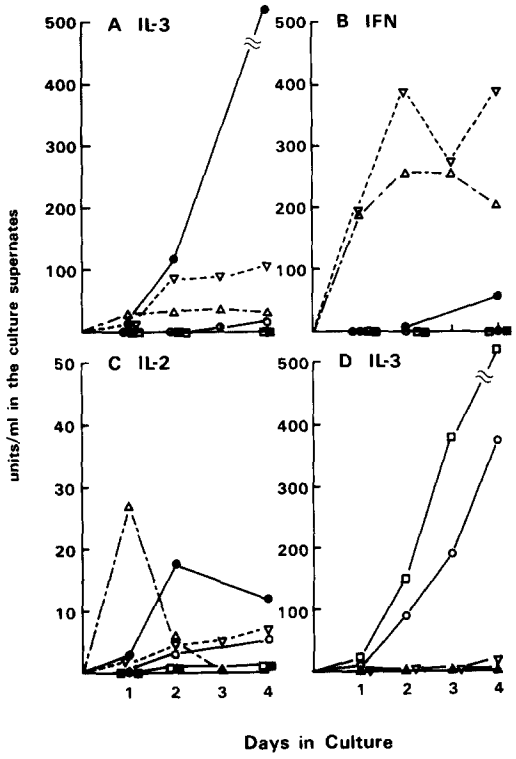

FIGURE 5. Production of lymphokines by splenic cells from mice infected with Mo-MLV. Spleen cells from normal BALB/c, +/+ mice (—○—), normal BALB/c, *nu/nu* mice (—□—), BALB/c, +/+ mice inoculated with Mo-MLV (—●—), and BALB/c, *nu/nu* mice inoculated with Mo-MLV (—■—), were cultured in the complete medium without any additional stimulant. For the comparison, spleen cells from normal BALB/c, +/+ mice were cultured with either 2 μg/ml of Con A (--△--) or 1 μg/ml of PHA (--▽--). Varying days after the culture, supernatants were harvested and assayed for IL-3 (*A*), IFN (*B*), and IL-2 (*C*) activity as described in Materials and Methods. In one experiment (*D*), spleen cells from Mo-MLV-infected BALB/c, +/+ mice were first treated with C′ alone (○), anti-Thy-1.2 + C′ (△), anti-Lyt-1.2 + C′ (▽), or anti-Lyt-2.2 + C′ (□). They were then similarly cultured in the plain medium, and IL-3 activity in the culture supernatants was assayed.

Mo-MLV infection, either live or UV inactivated, did not affect NK activity per se in the spleen at all, whereas IFN-inducer poly(I:C) significantly augmented NK activity (data not shown). From poly(I:C)-infected mouse spleen cells, however, continuous LGL lines could not be established by IL-2 alone. In the culture supernatants of spleen cells from Mo-MLV-infected mice, on the other hand, a surprising amount of IL-3 activity was detected without any additive stimulation in vitro (Fig. 5*A*). Conforming to the observation that no continuous lines could be established from both nude and scid mice, IL-3 production was not detected in the spleen cell culture of these T cell–deficient mice after the MLV infection (Fig. 5*A*). As shown in Fig. 5*D*, IL-3 activity preferentially derived from T cells of inducer phenotype, Thy^+,$Lyt\text{-}1^+$,$Lyt\text{-}2^-$. It was particularly noted that IL-3 production by Mo-MLV-infected splenocytes was rather selective over other lymphokines, such as IL-2 and IFN, as compared with the cases of mitogen-stimulated splenic cultures (Fig. 5, *B* and *C*).

Establishment of Continuous LGL Lines from Normal Splenic Cells In Vitro by Combined IL-3 and IL-2 Stimulation. To directly investigate the involvement of IL-3, an attempt was made to generate cell lines from normal spleen cells by combined stimulation with rIL-3 and rIL-2 entirely in vitro. As a source of rIL-3, serum-free culture supernatant of IL-3 gene-transfected COS-1 cells was used. As shown in Fig. 6, the COS-1 supernatant as well as WEHI-3 conditioned medium (CM) supported the proliferation of IL-3-dependent FDC-P2 cells in a dose-dependent fashion, while supernatant of GM-CSF gene-transfected COS-1

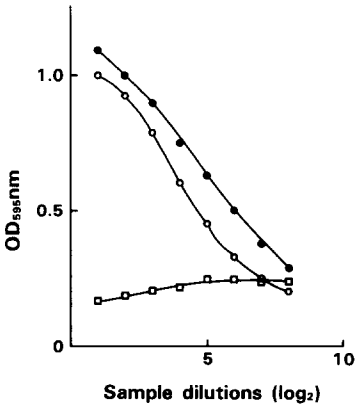

FIGURE 6. Dose-response titration curves of the IL-3 gene-transfected COS-1 cell supernatants on the proliferation of FDC-P2 line. Murine IL-3 and GM-CSF genes were cloned from a cDNA library of a T cell line (1H5.5) and transfected into COS-1 cells using the pcD-X vector as described in Materials and Methods. Serum-free conditioned media of COS-1 cells transfected with the IL-3 gene (●), GM-CSF gene (□), as well as WEHI-3 cells (○) were serially diluted and added to FDC-P2 cells (10^4 cells/well). 3 d later, the proliferation of FDC-P2 cells was assessed using MTT colorimetric assay system. Although not shown here, none of the conditioned media showed any detectable IL-2 activity as assayed on CTLL-2 cells.

TABLE III

Generation of Continuous Cell Lines In Vitro from Normal Splenic Cell Cultures by the Combined Stimulation with rIL-3 and rIL-2

Exp.	Strains of mice	Stimulation with: Primary culture (7 d)	Amount	Secondary Culture (≥7 d)	Amount	Line (number positive per total cultures)
			U/ml		*U/ml*	
I	BALB/c, +/+					0/6
		IL-2	100	IL-2	100	0/6
		IL-3	100	IL-3	100	0/6*
		IL-2	100	IL-3	100	0/6
		IL-3	100	IL-2	100	4/5
II	BALB/c, *nu/nu*	IL-3	100	IL-2	100	0/6
	CB-17, *scid/scid*	IL-3	100	IL-2	100	0/4
III	BALB/c, +/+	IL-2	100	IL-2	100	0/3
		IL-3	0.1	+ IL-2	100	0/3
		IL-3	1	+ IL-2	100	2/3
		IL-3	10	+ IL-2	100	3/3

In Exps. I and II, spleen cells from normal mice were cultured in the absence or presence of either rIL-2 or rIL-3 for 1 wk. The cultures were then fed with either homologous or heterologous lymphokine latter on every other day. The cultures were monitored for at least 3 wk, and scored positive when propagatable cell growth was obtained after splittings. In Exp. III, the cultures of normal spleen cells were initiated in the simultaneous presence of rIL-2 (100 U/ml) and various doses of rIL-3 (0–10 U/ml), with subsequent feedings with rIL-2.

* In these cultures, rather persistent proliferation of mast cells was observed. They could be propagated for substantial periods but regularly died off in 1–3 mos.

cells did not stimulate the FDC-P2 growth at all. Either rIL-3 (100 U/ml) or rIL-2 (100 U/ml) alone could initiate some proliferation of normal splenic cells, but it was transient and the cells regularly died off (Table III). On the other hand, when normal spleen cells were first stimulated with rIL-3 (100 U/ml) for a week and then kept fed with rIL-2 (100 U/ml), which mimicked the situation described, IL-2-dependent continuous cell lines could be established. The generation of

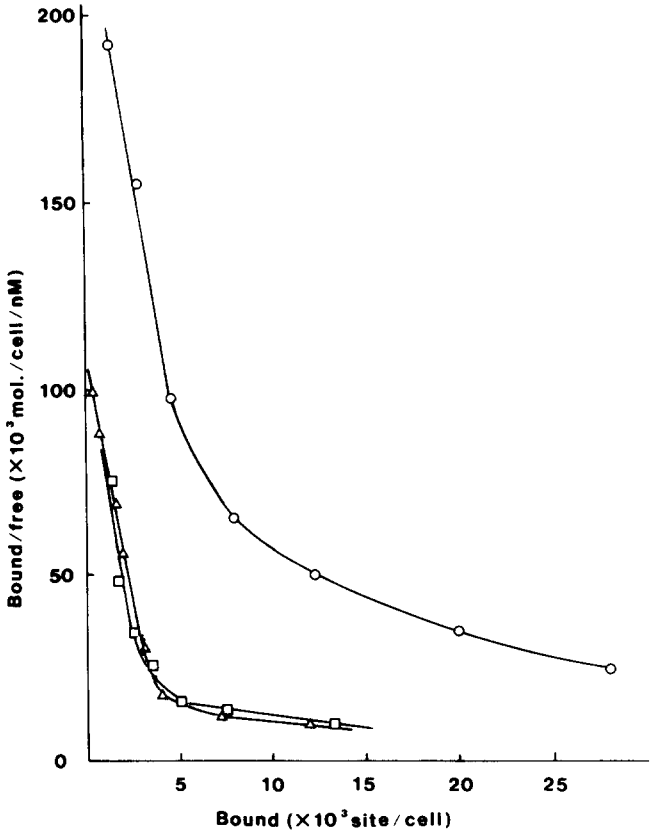

FIGURE 7. Scatchard plot analysis of cellular ^{125}I-IL-2 binding on LGL lines. LGL lines established by rIL-2 from Mo-MLV-infected mice, SPB2.4 (○), SPB-T3 (□), and a line generated from normal spleen cells by the combined rIL-3 and rIL-2 stimulation, 6B (△), were washed in cold medium, and then IL-2 binding assay was performed as described in Materials and Methods.

lines was dependent on the initial doses of rIL-3, and <1 U/ml of rIL-3 was apparently insufficient for the effect (Table III). A similar LGL line (6B) was also established starting with the normal splenic population that had been nylon wool purified and then treated with anti-Thy-1 plus complement. The phenotype of the 6B line, however, was again Thy-1$^+$. Reverse sequence of stimulation, that is rIL-2 first and then rIL-3, did not result in the development of cell lines. All of the cell lines thus established without any involvement of exogenous retroviruses showed identical characteristics morphologically, phenotypically, and functionally to the lines developed from MLV-infected mice.

The results thus supported the idea that MLV infection in mice induced strong IL-3 production in vivo, which in turn stimulated the LGL to unlimited proliferate by the stimulation with IL-2 in vitro.

Constitutive Expression of High-affinity IL-2-R on LGL Lines. One of the major factors limiting the persistent proliferation of normal splenic cells by IL-2 is suggested to be the decline of functional or high-affinity IL-2-R on the cells, which can be induced and/or maintained by other factors, such as specific antigens, mitogens, or by accessory cells. Unlike this situation, rIL-2 alone was sufficient for continuous proliferation of LGL in the spleen cells, which had been either infected in vivo with Mo-MLV or pretreated in vitro with rIL-3. We thus examined the expression of IL-2-R on the LGL lines. As shown in Fig. 7, the

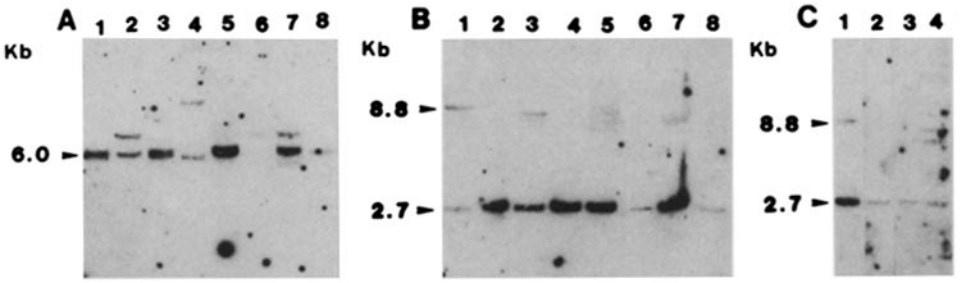

FIGURE 8. Southern blot analysis of DNA from LGL lines using a Cβ gene probe of TCR. 10 μg of DNA from LGL lines generated from Mo-MLV-infected mice were digested with Pvu II (*A*) or Hind III (*B*), electrophoresed, transferred to a filter, and hybridized with a ^{32}P-labeled Cβ probe (86T5). (*1*) liver, (*2*) SPB2.4, (*3*) SPB-3, (*4*) SPB-V1, (*5*) MLV/BS, (*6*) SPB-T2, (*7*) SPB-T3, and (*8*) SPB-T5. 10 μg of DNA from LGL lines developed from normal mice by in vitro combined stimulation with rIL-3 and rIL-2 were similarly analyzed for Cβ gene rearrangement after Hind III digestion (*C*). (*1*) liver, (*2*), 6B, (*3*) L3P-B1, and (*4*) L3P-B2.

LGL lines showed constitutive expression of high-affinity-IL-2-R (4–7 × 10^3/cell, K_d = 0.02 nM) in addition to low-affinity IL-2-R (K_d = 2 nM).

The LGL Lines are Genetically Committed to T Cell Lineage. Final question was then what was the exact cellular lineage of the LGL lines developed from MLV-infected or IL-3-primed mice. For this purpose, TCR genes were analyzed by Southern blot hybridization using the Cβ probe. As shown in Fig. 8, the Cβ gene of TCR was rearranged in all of the lines examined, including those derived from Mo-MLV-infected mice (*A* and *B*) and from normal mice by in vitro combined rIL-3 and rIL-2 stimulation (*C*). Although these LGL lines had not been cloned, it was noted that each of the lines seemed quite clonal in terms of the Cβ gene rearrangement patterns, and the patterns were different from each other. The results indicated that the LGL lines thus generated were all committed genetically to T lineage of lymphocytes.

Discussion

IL-2 induces the limited proliferation of a portion of normal splenic lymphocytes, one of the major responsive populations being LGL (7, 8). However, the present study indicated that IL-2 alone initiated and maintained essentially unlimited proliferation of LGL from the spleen cells of adult mice infected with Mo-MLV, resulting in the continuous cell lines. All the IL-2-dependent cell lines were quite homogeneous and showed uniform characteristics in spite of no preselective procedures. They were morphologically lymphocytes with characteristic intracytoplasmic coarse granules (LGL), and their surface phenotype was Thy-1$^+$,Lyt-1$^-$,L3T4$^-$,Lyt-2$^-$,AsGM$_1$$^+$,FcRγ$^+$ without exception. These features corresponded best to a set of NK cells, and indeed most of the lines did show NK-patterned cytotoxic activity. Furthermore, the lines from beige mice showed aberrant intracytoplasmic granules and no cytotoxic activity against NK-susceptible target cells. Southern blot analysis indicated that the Cβ gene of TCR had been rearranged in all LGL lines, and in at least one LGL line, full-length mRNA could be detected (20), indicating that the lines were genetically committed to T cell lineage. We have previously reported (1) that so-called NK cells consisted of

rather heterogeneous cell populations, and that some of them showed certain T cell–like features. Indeed, some of the LGL clones mediating NK activity were shown to have rearranged TCR genes both in man and mice (20, 22, 23), while the majority of endogenous NK cells were reported to have germline pattern (24, 25). It seems that MHC-unrestricted cytotoxic activity can be mediated by distinct populations of LGL, some genetically committed to T cell lineage while others are not, the terminology as well as interrelationship of each still a matter of argument.

Since the LGL lines were mostly monoclonal or oligoclonal as far as the Cβ rearrangement pattern was concerned, we first suspected that Mo-MLV infection was somehow directly involved in their development. However, no evidence of retroviral replication or proviral integration was obtained, as judged by RT assay and Southern blot analysis using a Mo-MLV-specific cDNA probe, suggesting the primary involvement of host factors induced by Mo-MLV infection for the generation of lines. It then became evident that spleen cells from Mo-MLV-infected mice, when incubated alone without any specific additive, produced surprising amounts of IL-3. IL-3 activity was found to derive from a Thy-1$^+$,Lyt-1$^+$,Lyt-2$^-$ population of spleen cells, and no such activity was detected in both BALB/c nude and CB.17 scid mice after Mo-MLV infection. Using the tumor-regressing mice infected with Mo-MSV/Mo-MLV, it was first reported by Ihle et al. (26) that IL-3 was produced by the specific immune T cells (Thy-1$^+$,Lyt-1$^+$,Lyt-2$^-$) in response to Mo-MLV-gp70 in vitro. In the present study, no viral antigen was added in vitro, but it was probable that viruses were brought into the culture in association with splenic cells other than LGL (Table II). Subsequently, Palacios (27) reported that Thy-1$^+$,Lyt-1$^+$,Lyt-2$^-$ cells from MRL/*lpr* mice spontaneously produced significant amounts of IL-3 in vitro, implicating the possible involvement in the development of generalized lymphoproliferation in the mice. It seems possible that retroviruses are somehow responsible for continuous IL-3 production in this strain of mouse. That IL-3, but not the virus itself, was primarily responsible for the unlimited proliferation of LGL by IL-2, has been confirmed by the observation that essentially identical LGL lines could be generated by rIL-2 alone from the normal spleen cells that had been precultured in vitro with rIL-3 (Table III).

IL-3 was originally described as a blastogenic factor for lymphoid cells (28). More recently, however, the major stress of the functional aspect of IL-3 seems to reside in the activity on hematopoietic cells as multipotential colony-stimulating factor (CSF) (29, 30). IL-3 was shown to induce hematopoietic stem cell colonies in vitro from the spleen cells of mice pretreated with 5-FU (31), and we have evidence that similar LGLs with NK activity can directly generate from IL-3-induced multipotential stem cell colonies in vitro (Minato, N., and T. Suda, manuscript submitted for publication). This suggests that IL-3 directly acted on a progenitor cell population from which LGL were selectively induced by IL-2. Once the lines had been established, however, LGL proliferated in response to only IL-2 but no longer to IL-3. In terms of lymphoid lineage, it was first reported by Happel et al. (32) that IL-3 initiated the growth of a mature inducer type of T cells (Thy-1$^+$,Lyt-1$^+$,Lyt-2$^-$) resulting in factor-independent growth.

More recently, a subpopulation of pre-B cells, but not mature B cells, in bone marrow was also reported to proliferate in response to IL-3 (33).

It was reported (34) that spleen cells of nude and scid mice have comparable or even increased NK activity. In this context, it was rather unexpected that LGL lines could not be established from these mice after Mo-MLV infection. This could not be explained by the fact that these mice lacked mature T cells to produce IL-3 in vivo after Mo-MLV infection, since sufficient IL-3 supplement in vitro followed by IL-2 did not result in LGL lines either. Our preliminary results indicate that some, but not all, of the present LGL lines express T3 (CD3) molecules in the absence of mature T cell markers. $T3^+$,$L3T4^-$,$Lyt\text{-}2^-$ cells represent the majority of fetal thymocytes as well as a minor population of adult thymocytes, and a recent report (35) indicates that the cells with the same phenotype also exist in small numbers in the periphery. Furthermore, a population of $T3^+$,$L3T4^-$,$Lyt\text{-}2^-$ fetal thymocytes could be induced by IL-2 to develop into LGL with NK-like activity (35a). Some of $T3^+$,$L3T4^-$,$Lyt\text{-}2^-$ cells have recently been reported (36, 37) to have another TCR-like structure distinct from the α/β complex. We thus feel that Mo-MLV infection or IL-3 combined with IL-2 selectively induces a special category of T cell lineage LGL endowed with NK activity. It is certainly unrealistic to attribute NK activity to a single category of cells, and the present results raise a good possibility that some, if not all LGL with NK activity in euthymic mice have differentiation pathways distinct from the majority of those in athymic mice.

LGL lines derived from Mo-MLV-infected spleens as well as IL-3-prestimulated normal spleen cells constitutively expressed large numbers of high-affinity IL-2-R. It thus seems most probable that unlimited proliferation of LGL from IL-3-primed spleen cells by IL-2 alone was due at least partly to the constitutive expression of functional IL-2-R. Recently, it was reported (38) that IL-3 induced IL-2-R expression at the transcriptional level in IL-3-dependent myeloid cell lines, although the IL-2-R was of low-affinity entity and apparently not functional (38). The exact effect of IL-3 on functional IL-2-R in the lymphoid lineage of cells remains to be elucidated.

Finally, what then would the physiological significance of the phenomenon be? The first concerns the regulatory role of LGL in hematopoiesis. In certain situations such as Mo-MLV infection, abundant and rather persistent production of IL-3 might result in rapid expansion of all the series of hematopoietic cells. On the other hand, NK cells were shown to be able to downregulate normal hematopoiesis (3, 4), and our previous results (9) indicated that cloned LGL lines were indeed cytotoxic to normal bone marrow cells as well. Recently, for instance, it was reported (39) that some diseases characterized by peripheral pancytopenia, including adult cyclic neutropenia, were associated with monoclonal expansion of LGL with the rearranged $C\beta$ gene. Thus, it can be presumed that LGL induced by IL-3 might provide an internal negative feedback mechanism for simultaneously accelerated hematopoiesis. The second concerns the possible involvements specifically in Mo-MLV-leukemogenesis. Mo-MLV preferentially induces leukemia of T cell lineage (40), although the exact mechanism of leukemogenesis is not completely clear. It was noted that all of the LGL lines were free from virus infection, whereas the viral infection was clearly detected

in the whole splenic population from which they originated. Thus, another possibility would be that the LGL population might play a significant role in the resistance of host to the exogenous retroviral infection, most likely through their NK activity, escaping viral infection themselves. Infection of adult mice with Mo-MLV rarely results in leukemogenesis, while leukemia reproducibly develops in newborn mice (41). Our preliminary experiments failed to generated LGL lines from Mo-MLV-infected newborn mice, implying this possibility. Using a cloned line, it was reported that LGL could protect thymomagenesis by split-dose radiations in mice, in which retroviral involvement was suspected (42). The investigation on the role of LGL in viral leukemogenesis is currently underway.

Summary

Continuous cell lines could be reproducibly established by culturing spleen cells from adult mice injected with MLV-producer cells or directly infected with Mo-MLV with rIL-2, whereas the culture of normal splenic cells with rIL-2 induced only transient and limited proliferation resulting in no such lines. All of the lines showed morphological characteristics as LGL with Thy-1$^+$,Lyt-1$^-$,L3T4$^-$,Lyt-2$^-$,AsGM$_1^+$,FcRγ^+ phenotype without exception, and most of them exhibited typical NK-patterned cytotoxicity. Analysis of reverse transcriptase activity of the culture supernatants as well as Southern hybridization of the DNA from the lines using an Mo-MLV-specific cDNA probe indicated no evidence of retroviral replication or proviral integration, suggesting that the generation of cell lines reflected a reactive process and viral infection was not directly responsible. It was subsequently revealed that Thy-1$^+$,Lyt-1$^+$,Lyt-2$^-$ spleen cells from mice infected with Mo-MLV in vivo spontaneously produced surprising amounts of IL-3 in vitro, leading to the possibility that IL-3 was responsible for the generation of lines. The possibility was directly supported by the observation that continuous lines with identical characteristics could be generated completely in vitro by sequential stimulation with rIL-3 and rIL-2 from normal spleen cells without any involvement of Mo-MLV. The Cβ gene of TCR was shown to be rearranged in all the lines examined, indicating the LGL lines were all genetically committed to T cell lineage. Unlike the situation in normal splenic populations expanded by rIL-2, where the expression of IL-2-R was progressively lost, constitutive expression of high-affinity-IL-2-R was observed in all the lines and thus, this was considered to explain the unlimited proliferation of them in response to rIL-2 alone. These results suggested the probable role of IL-3 in the regulation of growth and differentiation of a set of LGL committed to T cell lineage. The possible implications of the phenomenon in the regulation of hematopoiesis as well as in the control of Mo-MLV-induced leukemogenesis were discussed.

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