# DIFFERENTIATION IN VITRO OF T3$^+$ LARGE GRANULAR LYMPHOCYTES WITH CHARACTERISTIC CYTOTOXIC ACTIVITY FROM AN ISOLATED HEMATOPOIETIC PROGENITOR COLONY

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Large granular lymphocytes (LGL)[1] represent a population of lymphoid cells morphologically and functionally distinct from conventional T and B lymphocytes (1), but their exact progenitors as well as differentiation pathway remain largely unclear. We have recently observed that continuous LGL lines could be efficiently generated in vitro by IL-2 from the spleen cells of mice infected with Moloney murine leukemia virus (Mo-MLV) (2). Further analysis revealed that it was IL-3 induced by the retrovirus infection rather than virus itself that was responsible for the phenomenon (2). Thus, it was indicated that the sequential stimulation of normal spleen cells with IL-3 followed by IL-2 in vitro resulted in the selective and continuous proliferation of LGL (2). These observations facilitated our investigation of the role of IL-3 in the early differentiation of LGL.

IL-3 is shown to have a number of biological activities, including mast cell growth-promoting activity (3), granulocyte (G)/macrophage (M) colony-stimulating activity (3), pre-B cell–stimulating activity (4), and activity to induce an enzyme 20$\alpha$-hydroxysteroid dehydrogenase in T-lineage lymphocytes (5). Furthermore, evidence indicates that IL-3 is also involved in the proliferation of pluripotential hematopoietic progenitor cells. It was reported by Suda et al. (6) that IL-3 induced a new class of murine multipotential hematopoietic colonies, termed blast colonies, which had extremely high secondary plating efficiencies in vitro. In the present study, a direct attempt has been made to generate lymphoid cells from such multipotential hematopoietic colonies. The results definitely indicated that LGL with characteristic cytotoxic activity could directly be generated from the isolated hematopoietic progenitor cell colonies. Genetic analysis of TCR genes of the LGL revealed that both $\gamma$ and $\beta$ chain genes of TCR were rearranged with abundant mRNA for the $\gamma$ chain gene and much

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[1] *Abbreviations used in this paper:* 5-FU, 5-fluorouracil; LGL, large granular lymphocytes; Mo-MLV, Moloney murine leukemia virus; PEC, peritoneal exudate cells; rEPO, recombinant erythropoietin; rGM-CSF, recombinant granulocyte/macrophage colony-stimulating factor.

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lesser amount of mRNA for the $\beta$ chain gene. Based on these observations, an early differentiation pathway of a set of LGL will be discussed from an aspect of possible extrathymic, alternative differentiation of T-lineage lymphocytes.

## Materials and Methods

*Mice.* $BDF_1$(C57BL/6 × DBA/2 $F_1$) and BALB/c mice were purchased from Shizuoka Laboratory Animal Center, Shizuoka, Japan.

*Cell Lines.* Leukemic cell lines (YAC-1, RL$\delta$1, EL4, T3C12) were maintained in RPMI 1640 supplemented with 2 mM glutamine, antibiotics, $5 \times 10^{-5}$ M 2-ME, 10 mM Hepes, 1 mM sodium pyruvate, and 10% FCS (complete RPMI). T3C12 is an erythroid leukemic cell line induced by Friend leukemia virus, and was provided by Dr. Y. Ikawa, Physical and Chemical Institute, Tsukuba, Japan. FDC-P2 (7), an IL-3-dependent promyelocytic cell line, was maintained in complete RPMI supplemented with 100 U/ml rIL-3. DA-1 (8) and NFS-60 (9), myelogenous leukemic lines induced by Moloney-MLV and Cas-Br-M ecotropic virus, respectively, were cultured in complete RPMI with 100 U/ml rIL-3 and recombinant murine GM colony-stimulating factor (rGM-CSF), respectively. SPB-2.4 is a LGL line established from the spleen cells of BALB/c mice infected with Mo-MLV in vivo with subsequent stimulation in vitro with IL-2 as described before (2), and was maintained in complete RPMI supplemented with 100 U/ml human rIL-2. IL-2-dependent CTL line (CTLL-2) was cultured with complete RPMI supplemented with 100 U/ml rIL-2.

*Cytokines.* Recombinant human IL-2 (rIL-2) was provided by Shionogi Pharmaceutical Co. (Osaka, Japan), and the specific activity was $10^7$ U/mg protein. Murine rIL-3 was obtained from the serum-free culture supernatants of COS-1 cells transfected with a cloned IL-3 gene using pCD-X vector as described before (2), specific activity being $10^5$ U/mg protein. Titers of rIL-2 and rIL-3 were assayed using CTLL-2 and FDC-P2 as indicator cells, respectively. Murine GM-CSF gene was cloned from an inducer T cell line (1H5.5) as described before (10). The gene was transfected into COS-1 cells using pCD-X vector, and the serum-free culture supernatants of the transfected cells were used as rGM-CSF. The specific activity was $10^5$ U/mg protein. Human recombinant erythropoietin (rEpo) was provided by Chugai Pharmaceutical Co. Ltd., Tokyo, Japan. It had a specific activity of $1.7 \times 10^5$ U/mg protein by the in vivo assay using polycythemic mice. Murine rIL-4 was kindly supplied by Dr. H. Karasuyama, Tokyo University, Tokyo. It was obtained as culture supernatants of Ig-nonproducing myeloma line, X63-Ag8-653, transfected with a cloned IL-4 cDNA in a high-copy expression vector. IL-4 activity was assayed as T cell growth factor activity (11) on an HT-2 T cell line, and the titer was 8,000 U/ml.

*Blast Cell Colonies.* Normal BDF1 mice were injected intravenously with 5-fluorouracil (5-FU) (150 mg/kg), and 4 d later the spleens were removed and the cells were cultured in the semisolid culture medium containing 100 U/ml rIL-3 ($6 \times 10^5$ cells/35 mm dish). The culture medium was $\alpha$ medium supplemented with 1.2% methylcellulose (Fisher Scientific Co., Pittsburgh, PA), 30% FCS, 10 mg/ml deionized BSA (Sigma Chemical Co., St. Louis, MO), and $10^{-4}$ M 2-ME. The cultures were incubated for 6 d in a $CO_2$ incubator to develop colonies. For the secondary cultures, the blast colonies were individually lifted up with a 3-$\mu$l Eppendorf micropipette under the inverted microscopy, resuspended in 0.1 ml of $\alpha$ medium, and the cells of each single colony were recultured in methylcellulose medium in the presence of 100 U/ml rIL-3 and 1 U/ml rEpo. 8 d later, the numbers of various types of colonies were scored as described before (6). Plating efficiency was on average 59% (25–86%).

*Generation of LGL.* IL-3-induced blast colonies from the spleen cells of 5-FU treated mice were individually lifted up, resuspended, and cultured in the complete RPMI supplemented with 100 U/ml rIL-2 in the flat-bottomed microtiter wells (one colony/well), which had been precoated with resident peritoneal adherent cells irradiated with 5,000 rad of x ray. The cultures were fed with rIL-2 every other day, and monitored for 3 wk. Cells outgrown in the wells were further expanded in the presence of both rIL-2 and irradiated peritoneal exudate cells (PEC).

*CD3 mAb.* Hamster anti-mouse T3 mAb (2C11) was kindly provided by Dr. J. Bluestone, National Institutes of Health, Bethesda, MD. The hybridoma culture supernatants (1% FCS) were purified with protein A–Sepharose 4B column. Normal hamster IgG was similarly purified by the same column.

*Flowcytometry Analysis.* Surface phenotypes of cells were analyzed using FACS402 (Becton Dickinson & Co., Mountainview, CA). mAbs used included, anti-Thy-1.2 (mouse IgM), anti-T3 (2C11, hamster IgG), anti-L3T4 (GK1.5, rat IgG), anti-Lyt-2.2 (mouse IgM), anti-IL-2-R (PC.61, a gift from Dr. J. Yodoi, Kyoto University, Japan), anti-B220 (14.8; a gift from Dr. Nishikawa, Kumamoto University, Japan), and anti-T200 (rat IgG). Purified rabbit anti-asialo GM1 (GA1) was provided by Dr. S. Habu, Tokai University, Japan. Cells were incubated with either medium alone or various antibodies at 4°C for 30 min, washed, and then incubated with the corresponding FITC-conjugated second antibodies at 4°C for another 30 min before the analysis by FACS.

*Cytotoxicity Assay.* Cytotoxic activity of the cells was assayed using the 6-h $^{51}$Cr-release method, and percent specific $^{51}$Cr release was calculated as described before (2). When factor-dependent cells were used as targets, relevant factors (either rIL-3, rGM-CSF, or rIL-2) were included in the assay culture medium to prevent the spontaneous cell death due to the lack of growth factors.

*Analysis of TCR Genes.* Rearrangement of TCR genes was examined by Southern hybridization as described before (12). Briefly, genomic DNA was isolated from liver cells and LGL lines. 10 μg DNA was digested with either Pvu II or Hind III for Cβ gene, or with Eco RI for Cγ gene analysis. DNAs were then fractionated on agarose gel, transferred to nitrocellulose filters, and hybridized with nick-translated $^{32}$P-labeled Cβ probe (an Eco RI fragment of clone 86T5, kindly provided by Dr. M. Davis of Stanford University, Stanford, CA), or Cγ probe (pHDS203, kindly provided by Dr. S. Tonegawa of Massachusetts Institute of Technology, Cambridge, MA). For Northern blot analysis, total cellular RNA was extracted from the cells as described before (12). 10-μg RNAs were treated with glyoxal and DMSO, electrophoresed on an agarose gel, and transferred to a nitrocellulose filter. The filter was hybridized with the $^{32}$P-labeled Cβ or Cγ probes.

## Results

*Generation of LGL from Isolated Hematopoietic Progenitor Colonies.* Overall protocol for the experiments is schematically illustrated in Fig. 1. Normal $BDF_1$ mice were injected with 5-FU (150 mg/kg i.v.), which was shown to result in the enrichment of primitive hematopoietic progenitors (13). 4 d later, the spleens were removed and the cells were cultured in the methylcellulose medium containing 100 U/ml rIL-3 ($6 \times 10^5$ cells/35 mm dish). On day 6 of the culture, small colonies consisting of blast cells with little sign of differentiation were observed to develop (Fig. 2, *A* and *B*). ~5–10 blast colonies, each consisting of 20–200 cells, could be identified in the dish that received $6 \times 10^5$ spleens cells. By lifting each blast colony on day 6 and replating individually into secondary methylcellulose culture in the presence of rIL-3 and rEpo, colonies consisting of various hematopoietic cells including granulocytes, macrophages, megakaryocytes, mast cells, and erythroblasts, now developed from every single blast colony. The representative series of experiments are summarized in Table I. The plating efficiency was generally >60%. A representative mixed colony is shown in Fig. 2, *C* and *D*. The results confirmed that IL-3 induced a unique type of colonies consisting of blast cells from the spleen cells of 5-FU-treated mice, most of which were mono- or multipotential progenitors for hematopoietic cells.

A direct attempt was then made to generate LGL from isolated blast colonies in vitro. We have already reported that optimal proliferation of LGL from normal mice required both IL-2, a primary growth factor, and the contact with

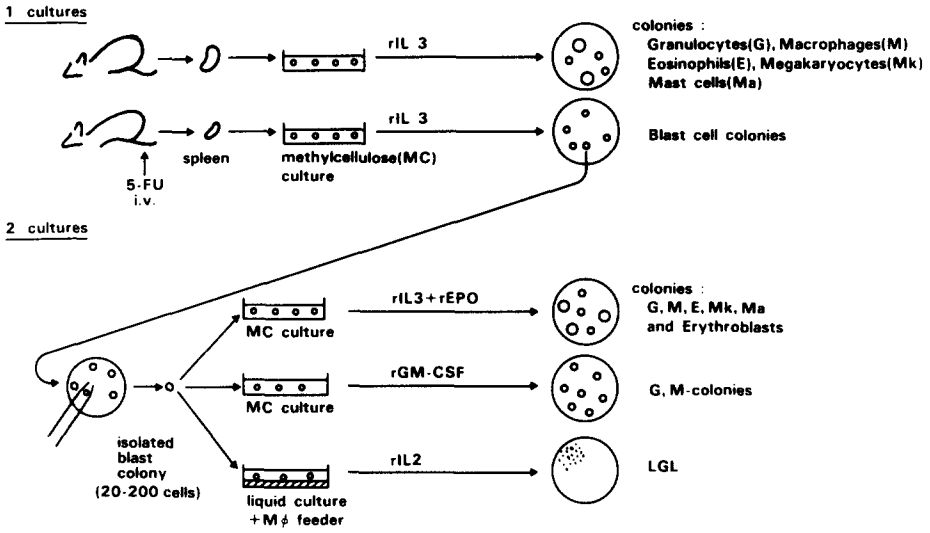


FIGURE 1. Schematic illustration of an over-all experimental protocol for generating LGL from an isolated IL-3-induced blast colony. The results of the secondary culture of isolated blast colonies with rGM-CSF are not shown in the present report, but have been reported in our previous report (10).

accessory macrophages to maintain the expression of functional IL-2-R (14). Thus, rIL-3-induced blast colonies on day 6 were individually lifted, resuspended, and cultured in the liquid medium in the presence of both rIL-2 (100 U/ml) and 5,000 rad irradiated PEC (see Fig. 1). The culture wells were fed with rIL-2 every other day. In 5 of 24 culture wells that received a single blast colony (on average 150 cells), the outgrowth of cells was observed in 10–14 d (Fig. 1*E*). Morphological examination revealed that they were quite homogenous large lymphoid cells with typical coarse azurophilic granules scattered in the cytoplasms, namely LGL, without exception (Fig. 1*F*). In no case was the generation of regular small lymphoid cells observed in the culture conditions.

The results thus clearly demonstrated that LGL could be directly generated from hematopoietic progenitor cells in vitro.

*Establishment of a Continuous Line of LGL Generated from a Single Hematopoietic Progenitor Colony.* LGL generated in the culture wells that received individual IL-3 blast colonies were further expanded in the new dishes in the presence of both rIL-2 and irradiated PEC. From one of the five positive wells, a continuous cell line (IL3B1) was successfully obtained. Morphologically, the IL3B1 line consisted of a homogeneous population of LGL indistinguishable from those in the primary culture well. As shown in Fig. 3, long-term proliferation of IL3B1 was observed only in the presence of both rIL-2 and irradiated PEC. rIL-2 alone supported a certain degree of the growth, but failed to maintain persistent proliferation in a few passages. Since the line was originated from a blast colony induced by rIL-3, the responsiveness of IL3B1 to rIL-3 was then examined. As is also shown in Fig. 3, IL3B1 showed no proliferative response to rIL-3 any more, even in the presence of irradiated PEC. Furthermore, rIL-4, which was also shown to have T cell growth factor (TCGF) activity (11), could not at all

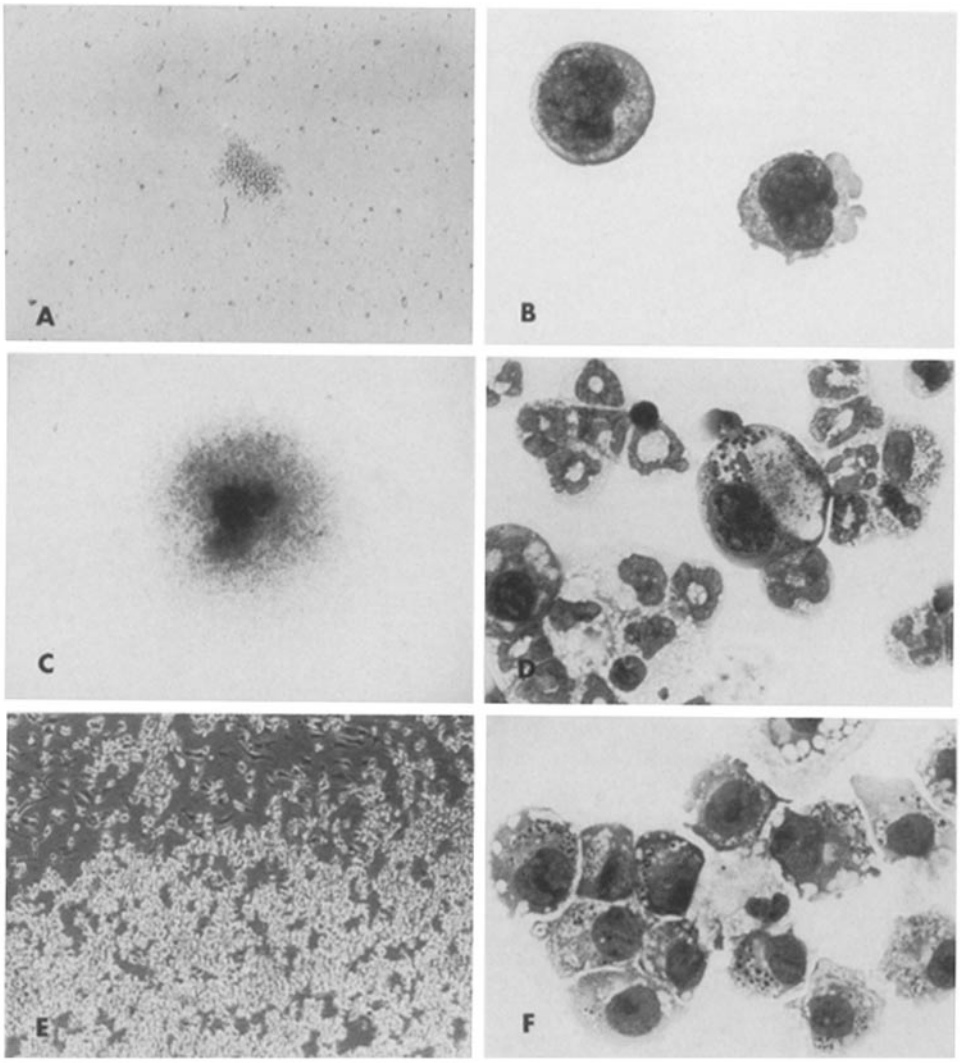


FIGURE 2. Morphology of the IL-3-induced blast colony and lymphohematopoietic cells derived thereof. (*A*) An IL-3-induced blast colony on day 6 generated from the spleen cells of mice pretreated with 5-FU as described in Materials and Methods (× 40), and (*B*) May-Grünwald-Giemsa staining of the cytospun preparation of the blast colony shown in *A* (× 400). (*C*) A mixed colony generated from the secondary culture of an IL-3-induced blast colony in the presence of rIL 3 and rEpo (× 16), and (*D*) May-Grünwald-Giemsa staining of the mixed colony (× 250). Note the difference in the magnification scales between *A* and *C*. Mature neutrophils, macrophages, megakaryocytes, and erythroblasts can be recognized in this particular mixed colony. (*E*) Phase contrast picture of the culture well that received an IL-3-induced blast colony in the presence of rIL-2 and 5,000 rad irradiated PEC 2 wk before (× 40). (*F*) May-Grünwald-Giemsa staining of the cells shown in *E* (× 250). These are apparently large lymphoid cells containing azurophilic granules in their cytoplasms (LGL). In the center of the picture is a macrophage used as a feeder layer. Original magnifications are indicated in parentheses.

TABLE I

*Secondary Colony Formation by Replating IL-3-induced Blast Colonies*

| Blast colony number | Cell numbers/colony | Types of colony* | | | | | | Total colony numbers | Plating efficiency |
|---|---|---|---|---|---|---|---|---|---|
| | | GM | B | Meg | Mast | GMM | GEMM | | |
| | | | | | | | | | % |
| 1 | 200 | 138 | 1 | 0 | 0 | 18 | 0 | 157 | 79 |
| 2 | 60 | 33 | 1 | 0 | 0 | 0 | 2 | 36 | 60 |
| 3 | 200 | 105 | 8 | 1 | 3 | 0 | 25 | 142 | 71 |
| 4 | 150 | 117 | 0 | 0 | 0 | 0 | 7 | 124 | 83 |
| 5 | 150 | 86 | 0 | 1 | 2 | 0 | 31 | 120 | 80 |
| 6 | 150 | 30 | 1 | 1 | 0 | 1 | 4 | 37 | 25 |
| 7 | 200 | 123 | 0 | 1 | 0 | 8 | 3 | 135 | 68 |
| 8 | 200 | 124 | 0 | 0 | 0 | 20 | 1 | 145 | 73 |
| 9 | 150 | 127 | 0 | 0 | 0 | 0 | 2 | 129 | 86 |

Blast colonies were developed from the spleen cells of 5-FU-pretreated BDF1 mice as described in Materials and Methods. On day 6 of the culture, individual blast colonies were lifted with a 3-$\mu$l Eppendorf micropipette from methylcellulose medium and resuspended in 0.1 ml $\alpha$ medium. Cells of each colony were added to the secondary culture dishes containing methylcellulose medium supplemented with 100 U/ml rIL-3 and 1 U/ml rEpo, and gently agitated to disperse cells. After 8 d of culture, the numbers of colony were scored under the inverted microscope. To confirm the cell lineage, colonies were lifted and examined for morphology by Giemsa staining.

* Abbreviations for colony types are as follows: GM, granulocyte/macrophage colonies; B, erythroid bursts; Meg, megakaryocyte colonies; Mast, mast cell colonies; GMM, granulocyte/macrophage/megakaryocyte colonies; GEMM, granulocyte/erythroid/macrophage/megakaryocyte colonies.

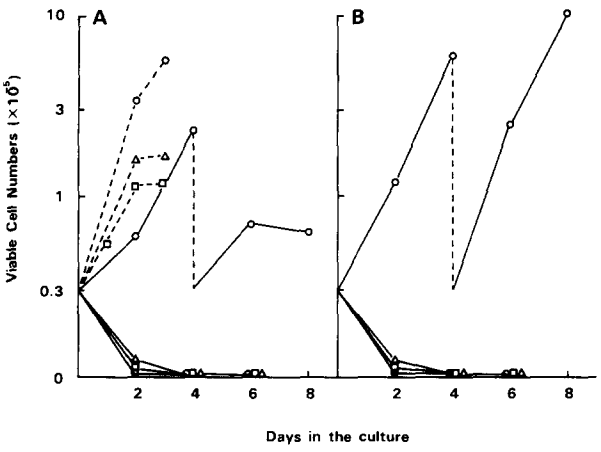


FIGURE 3. Effect of various recombinant growth factors on the proliferation of IL3B1 line. IL3-B1 cells ($3 \times 10^4$ cells/2 ml) were cultured in the absence (*A*) or presence (*B*) of $10^6$ irradiated normal peritoneal adherent cells. Various cytokines were added into the culture on day 0, and the viable cell numbers were counted on days 2, 4, 6, and 8. On day 4, cultures with growing cells were split into the new dishes at the original concentration. Growth factors included, 100 U/ml of rIL 2 (—○—), 100 U/ml of rIL 3 (—□—), and 100 U/ml of rIL-4 (—△—). As a negative control, cells were cultured with complete medium alone (—●—). As positive controls for each growth factor, the growth curves, of CTLL-2 in the presence of rIL 2 (--○--) or rIL 4 (--△--), and of NFS-60 in the presence of rIL 3 (--□--) are also shown.

support the proliferation of IL3B1 either, while it did stimulate the proliferation of a conventional T cell line (CTLL-2) (Fig. 3).

Thus, the proliferation of IL3B1 was strictly dependent on IL-2, but not on IL-3 nor IL-4, and for optimal growth the coexistence of normal macrophages was required.

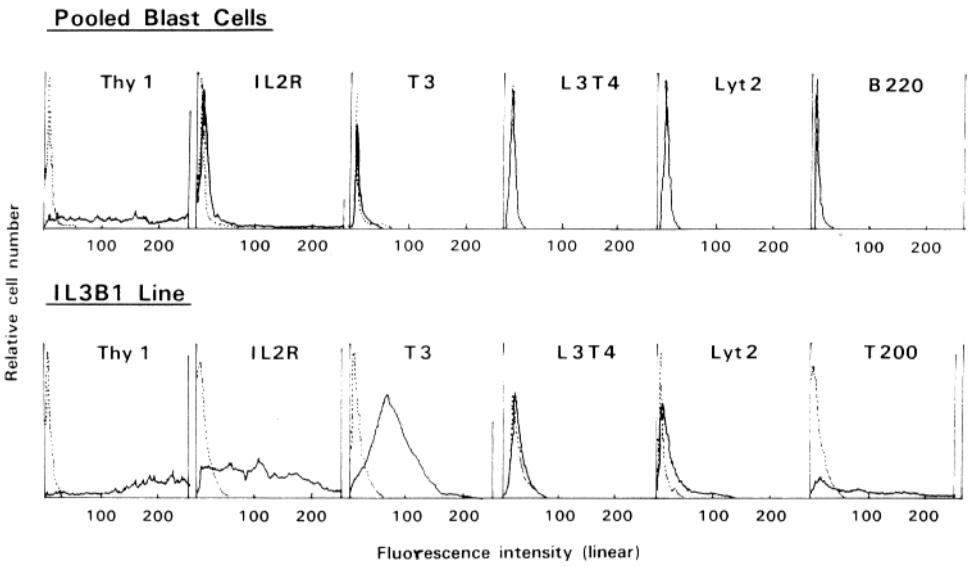


FIGURE 4. Flowcytometric analysis of pooled IL-3 blast cells and IL3B1 line derived thereof. IL-3-induced blast colonies were developed from 5-FU-treated mice as described in Materials and Methods. On day 6, ~$10^3$ of blast colonies, each consisting of 100–200 cells, were individually lifted up from 90 dishes in total. They were pooled and resuspended for the analysis. IL3B1 was a continuous LGL line, which originated from a single IL-3 blast colony and was propagated in vitro in the presence of both rIL-2 and irradiated PEC. Cells were incubated with various antibodies and analyzed with FACS402 as described in Materials and Methods. Dotted line indicates the negative control, in which cells were treated with the corresponding second antibody alone.

*Phenotypical Analysis of IL3B1 Line.* Further analysis was then performed using the IL3B1 line. Surface phenotypes of IL3B1 are summarized in Fig. 4, in comparison with pooled cells of day 6 blast colonies. Conforming to the report that hematopoietic stem cells bore Thy-1 antigen at various degrees (15), pooled blast cells did express Thy-1 antigen. Differentiation antigens for T cells (T3, L3T4, Lyt-2) and B cells (B220) were practically undetectable on these blast cells. On the other hand, IL3B1 cells derived thereof clearly expressed T3 in addition to Thy-1 antigen, while another Thy-$1^+$ LGL line, SPB-2.4, lacked T3 antigen (data not shown). Nonetheless, other antigens found on mature T cells, L3T4 and Lyt-2, were not detected at all or expressed only very marginally, respectively, on IL3B1. Actually, in the early phase after the establishment of the line, Lyt-2 antigen could not be detected at all, and then became marginally detectable as propagated in the culture (<5%). IL3B1 also expressed asialo GM1 (GA1) (data not shown). Rather surprisingly, they were strongly stained with both T200 and B220 mAb (data not shown.) Conforming to the IL-2 dependency, IL3B1 expressed IL-2-R, although at much less intensity than the IL-2-dependent CTL line, CTLL-2 (data not shown). It was also noted that a very minor proportion (~5%) of day 6 blast cells apparently expressed IL-2-R weakly.

It was thus indicated that LGL generated from blast colony were Thy-$1^+$,$T3^+$,$L3T4^-$,Lyt-$2^-$ $T200^+$ asialo $GM1^+$, with possible tendency to acquire Lyt-2 antigen marginally in the culture.

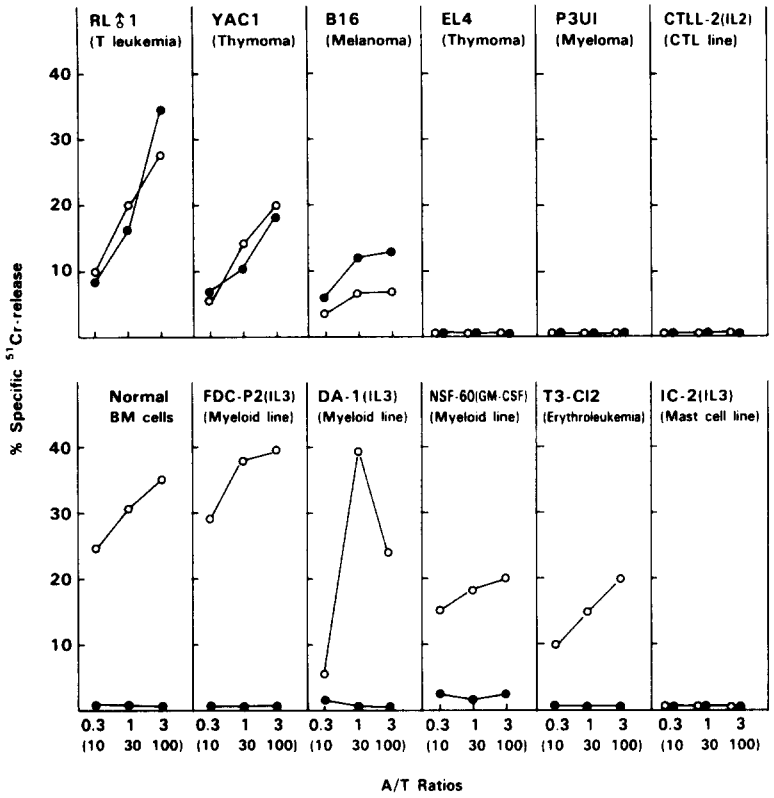


FIGURE 5. Cytotoxic activity of the IL3B1 line. Cytotoxic activity of the IL3B1 line (○) was examined in comparison with endogenous NK activity in freshly isolated normal spleen cells (●) against a panel of target cells. Effector-to-target ratios were 3:1, 1:1, and 0.3:1 for IL3B1; and 100:1, 30:1, and 10:1 for normal spleen cells. Target cells included representative NK-susceptible tumor lines (RL♂1, YAC1, B16), NK-resistant lines (EL4, P3U1, CTLL-2), normal bone marrow (BM) cells, and a number of myeloid cell lines (FDC-P2, DA-1, NFS-60, T3C12, IC-2). All of the myeloid cell lines were factor-dependent lines, each factor being indicated in the parenthesis. When these factor-dependent lines were used as targets, relevant growth factors were included in the cytotoxicity assay cultures. Cytotoxic activity was determined using the 6-h $^{51}$Cr-release method as described in Materials and Methods.

*Cytotoxic Activity of the IL3B1 Line.* One of the known major functions of LGL is non–MHC-restricted cytotoxic activity against various target cells, and thus cytotoxic activity of IL3B1 was examined. IL3B1 killed representative NK-susceptible tumor cells such as YAC-1, RL♂1, and B16 with a comparable pattern as freshly isolated normal splenic cells without affecting NK-resistant EL4 and P3U1 tumor cells (Fig. 4). In addition, the IL3B1 line exhibited significant cytotoxic activity against normal freshly isolated bone marrow cells, while fresh splenic cells hardly affected them. To delineate the latter cytotoxic activity, the effect of IL3B1 cells on various myeloid cell lines was then examined. As also shown in Fig. 5, IL3B1 cells displayed cytotoxic activity against most of these rather immature myeloid lines, including FDC-P2, DA-1, NFS-60, and T3C12. None of these cell lines were affected by freshly isolated spleen cells. FDC-P2, DA-1, and NFS-60 were factor-dependent cell lines, and the cytotoxicity assay

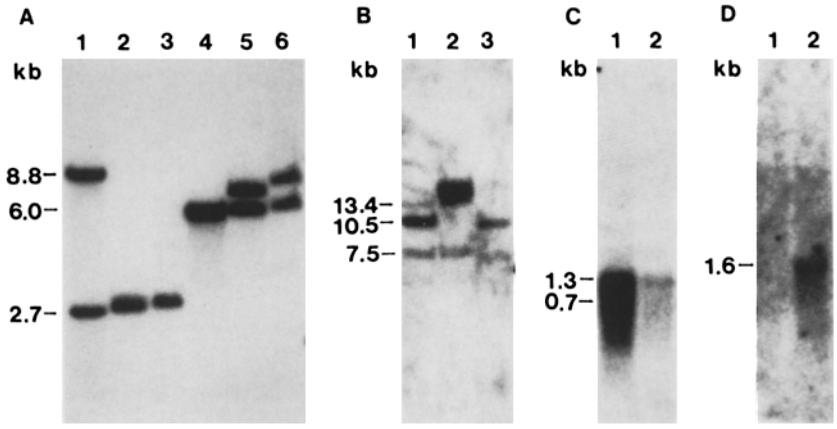


FIGURE 6. Analysis of TCR genes in the IL3B1 line. Rearrangement and mRNA of IL3B1 cells were examined in comparison with another LGL line, SPB2.4, established from a mass culture of Moloney leukemia virus–infected mice. (*A*) Southern blot analysis of DNA using a $\beta$ chain gene probe of TCR. DNAs were isolated from BDF1 liver cells (lanes *1* and *4*), IL3B1 cells (lanes *2* and *5*) and SPB2.4 cells (lanes *3* and *6*). 10 $\mu$g of each DNA was digested with either Pvu II (lanes *1–3*) or Hind III (lanes *4–6*), fractionated on agarose gel, transferred to nitrocellulose filter, and hybridized with nick-translated $^{32}$P-labeled C$\beta$ probe. (*B*) Southern blot analysis of DNA using a $\gamma$ chain gene probe of TCR. DNA was isolated from liver cells (lane *1*), IL3B1 cells (lane *2*), and SPB2.4 cells (lane *3*). They were digested with Eco RI, and similarly hybridized with C$\gamma$ probe. (*C*). Northern blot analysis of RNA using C$\beta$ probe. RNAs were extracted from SPB2.4 (lane *1*) and IL3B1 (lane *2*). 10 $\mu$g of total RNA was electrophoresed on agarose gel, transferred on nitrocellulose filter, and hybridized with $^{32}$P-labeled C$\beta$ probe. (*D*) Northern blot analysis using C$\gamma$ probe. The same filter used in C was washed and rehybridized with C$\gamma$ probe.

was performed in the medium containing relevant factors. Among them, the retrovirus-free FDC-P2 line, which represented progenitor cells committed to granulocytic differentiation with self-renewal potential (7), was consistently the most susceptible to the cytolysis by IL3B1. In contrast, a similarly IL-3-dependent P (mast) cell line (IC-2) and an IL-2-dependent CTL line (CTLL-2) were not affected by IL3B1 at all.

The results thus indicated that the IL3B1 LGL line was endowed with conventional NK activity, and further revealed that it had an additional spectrum of cytotoxic activity distinct from conventional NK activity, most likely directed toward certain stages of normal and continuous hematopoietic cells.

*Analysis of TCR Genes of the IL3B1 Line.* Since IL3B1 line clearly expressed T3 molecules, which are generally associated with TCRs, TCR genes were analyzed. DNA was extracted from IL3B1 and SPB2.4 as a control, digested with convenient restriction enzymes, electrophoresed on agarose gel, transferred on filters, and hybridized with $^{32}$P-labeled TCR probes. As shown in Fig. 6*A*, the $\beta$ chain gene was clearly rearranged. In Pvu II digestion, the 8.8-kb germline band was deleted, and in Hind III digestion, an extraband was detected above the 6.0 kb germline band. $\gamma$ chain gene of TCR was also rearranged in IL3B1 line (Fig. 6*B*). Thus, the 10.5-kb germline band was almost deleted and, instead, at least two overlapped extrabands were detected above the 13.4-kb germline band. The rearrangement patterns of these genes indicated that IL3B1 line was monoclonal. Both $\beta$ and $\gamma$ chain genes of the SPB2.4 LGL line were also

rearranged as already reported (12). We then examined the expression of mRNA for TCR in the IL3B1 line by Northern blot analysis. Conforming to the previous report (12), the SPB2.4 LGL line expressed full-length mRNA for the $\beta$ chain gene in the absence of any detectable mRNA for the $\gamma$ chain gene (Fig. 6, *C* and *D*). On the other hand, IL3B1 expressed abundant mRNA for the $\gamma$ chain gene. In terms of the $\beta$ chain gene, IL3B1 apparently transcribed full-length mRNA, but the signal was rather faint and much less than that of SPB2.4, when compared with the same dose (10 $\mu$g) of RNA loaded.

## Discussion

Present experiments were performed in an attempt to clarify the origin and differentiation of LGL in well-defined conditions in vitro. Day 6 colonies induced by rIL-3 from the spleen cells of mice pretreated with a high dose of 5-FU consisted of cells with quite homogeneous morphology with little sign of differentiation (blast cells) (Fig. 1). A small blast colony consisting of 20–200 cells is considered to have developed from a single multipotential progenitor cell. Thus, by the secondary cultures of individual blast colonies in the presence of rIL-3 and rEpo, essentially all components of hematopoietic cells developed with various proportions (Table I). The hematopoietic spectrum varied significantly from colony to colony, even with the same hematopoietic factor (rIL-3), suggesting the intrinsic diversity of the commitment of each progenitor colony. In addition, the nature of the factors also affected the hematopoietic spectrum of the blast colonies. We have already reported that the secondary culture of the same blast colonies in the presence of rGM-CSF resulted solely in GM colonies without other hematopoietic components (10). In any case, since the plating efficiencies of the blast colonies in the secondary cultures were quite high (on average 59%), most of the cells in the day 6 colony were considered to represent mono- and/or multipotential progenitors for hematopoietic cells.

The present results demonstrated that the direct progenitors for at least a set of LGL were included in the blast colony, proving that LGL originated from common hematopoietic progenitor cells. A few critical questions, however, remained to be resolved. It remained to be seen whether putative progenitors for LGL had been already committed to the lineage at the stage of day 6 colonies, or still remained multipotential for other hematopoietic cells, that is, whether the commitment occurred before or after the secondary specialized culture condition with IL-2. In this aspect, it was noted that a very minor portion of day 6 blast cells weakly expressed IL-2-R. Very recently, Palacios et al. (16) reported IL-3-dependent cell lines with TCR genes in germline configuration, which had a restricted potential to differentiate into T cells. After in vitro induction of differentiation by 5-azacytidine, the factor dependency for the growth of the pro-T cell clone was shown to switch from IL-3 to IL-2. The phenomenon resembles our present observation, in which a LGL line generated by IL-2 from an IL-3-induced blast colony responded only to IL-2 but not to IL-3 anymore (Fig. 3). Their finding may imply that preferential IL-2 responsiveness appears after the genetic commitment to the T cell lineage. If this is the case, it would be that the critical commitment to LGL, which belong apparently to T cell lineage, occurred during the 6-d primary culture period. This does not necessar-

ily contradict the assumption that the genetic manifestations per se, such as TCR rearrangement and T3 expression, might have occurred in the secondary culture with IL-2. Indeed, the essential role of the IL-2/IL-2-R system in the generation of TCR-bearing mature T cells in the fetal thymus has recently been reported using organ culture system (17). In this aspect, it is critical to know whether IL-2-R detected on a minor portion of day 6 blast cells are indeed functional, and this remains to be elucidated. Frequency of the committed LGL progenitors in the colony also remained uncertain. The monoclonal rearrangement patterns of TCR genes in the IL3B1 line suggest that the LGL line originated most likely from a single cell in some 200 cells of a blast colony. However, since the plating efficiency in the secondary liquid culture system is uncertain and most likely very low compared with hematopoietic colony assay, there is no clue for this at present.

Phenotypical analysis indicated that LGL generated from blast colony expressed T3 (CD3), in addition to Thy-1 antigen without differentiated T cell markers such as L3T4 and Lyt-2 (Fig. 4). Furthermore, analysis of TCR genes indicated that both $\beta$ and $\gamma$ genes of TCR were rearranged (Fig. 5). The results strongly suggested that these LGL were genetically committed to T cell lineage, phenotypically corresponding to the so-called $T3^+$ double-negative ($T3^+DN$) lymphocytes. Pooled cells in the day 6 blast colonies, on the other hand, expressed no detectable T3, and although direct genetic analysis of TCR in these cells was difficult because of extremely limited cell numbers, it seems most unlikely that they had rearranged TCR genes, considering their potential to develop into nonlymphoid hematopoietic cells as well. In either case, the present results indicate that the productive rearrangements of TCR genes as well as T3 expression could be induced utterly in vitro starting from a single putative hematopoietic stem cell in the absence of any thymic influence. It had been recognized that functional T cells, particularly class I MHC–restricted cytotoxic T cells, could be generated from the spleen cells of congenitally athymic nude mice when IL-2 was exogenously provided (18–20). More recently, it was also shown (21) that a portion of splenic cells from normal nude mice did show rearranged $\gamma$, $\beta$, and $\alpha$ genes of TCR and expressed full-length mRNA for these genes. These results indicate that a certain degree of T cell differentiation can proceed even in nude mice.

$T3^+$ double-negative ($T3^+DN$) lymphocytes represent the majority of fetal thymocytes (22) and are also present in a small proportion in the adult thymus (23), and thus they have been considered to be immediate precursors for functional T cells. More recently, however, $T3^+DN$ cells were reported to be present also in the peripheral tissues of normal adults, such as peripheral blood (23) and skin (24), in small numbers, and were shown to appear even in cerebrospinal fluid in certain demyelinating diseases (25). The origin of these peripheral $T3^+DN$ cells, as well as the relationship to intrathymic $T3^+DN$ cells, remains unclear at present. Recent analysis indicated that $T3^+DN$ cells in the fetal thymus and some of the peripheral tissues expressed the second TCR-like structure involving $\gamma$ chain gene products (26, 27). In the murine system, it was also recently reported that $T3^+DN$ LGL that originated from the skin (dendritic epidermal cells, DEC) expressed T3-associated TCR-like structures consisting of

a $\gamma/\delta$ heterodimer (28). In the present study, it was indicated that IL3B1 was $T3^+$, and expressed abundant full-length mRNA for $\gamma$ chain genes with much less mRNA for $\beta$ chain genes, although it is yet to be examined whether IL3B1 indeed expresses $\gamma$ chain protein associated with T3. Along with the observation that DEC were present in nude mouse skin as well (29), our present results support the idea that at least a portion of the peripheral $T3^+$DN population can arise in vivo in various tissues prethymically.

As far as we know, no evidence is available that these peripheral $T3^+$DN cells can further differentiate into mature classical T cells in any condition in vivo or in vitro. It seems possible that the present pathway may represent a unique extrathymic differentiation pathway of a minor set of T cell compartments independent from major T cell development in the thymus. The experiments to examine whether mature classical T cells can be generated from IL-3-induced blast colonies in the thymic environment in vitro are currently under way.

One of the major functions of fresh LGL is NK activity, and the IL3B1 line did show NK-like cytotoxic activity, affecting representative NK-susceptible target cells (Fig. 5). The detailed analysis of the cytotoxic spectrum, however, indicated that it was not necessarily the same as that of the fresh splenic NK activity. Thus, the IL3B1 line showed significant cytotoxic activity against freshly isolated BM cells as well as several immature myeloid cell lines, all of which were resistant to fresh splenic NK activity. Since $T3^+$DN cells represent only a very minor portion of the splenic NK population, such a discrepancy is not surprising. We and others have suggested that the TCR was not directly involved in the cytotoxicity against conventional NK target cells (11, 30); indeed, our preliminary results indicated that anti-T3 mAb did not affect the cytotoxicity of IL3B1 against the RL$\delta$1 target, supporting the idea. The effect of anti-T3 mAb on the cytotoxicity of IL3B1 on myeloid lines was complicated by the antibody-dependent cytotoxicity mediated by the Fc receptors on these targets (our unpublished observation), and thus it remains to be seen whether T3 molecules are involved in this type of cytotoxicity of IL3B1.

Recently the reports are accumulating on the diseases associated with either polyclonal or monoclonal increase in $T3^+$ LGL population with rearranged TCR genes (31, 32). Despite the heterogeneous nature of the diseases, one of their common clinical features is peripheral cytopenia, such as granulocytopenia, anemia, and thrombocytopenia (31–33). These results may imply that a $T3^+$DN set of LGL might function as one of the endogenous negative feedback mechanisms in the hematopoiesis in vivo. The present results that IL3B1 exhibited rather selective cytotoxic activity against normal bone marrow cells as well as immature myeloid cell lines are certainly consistent with the idea.

## Summary

Blast colonies were developed by rIL-3 from the spleen cells of mice pretreated with 5-fluorouracil (5-FU) in the methylcellulose cultures. When such IL-3-induced blast colonies were individually lifted up and recultured in the presence of rIL-3 and recombinant erythropoietin (rEpo), a variety of hematopoietic colonies developed from every single colony, including neutrophils, macrophages, eosinophils, megakaryocytes, mast cells, and erythroblasts. The results

indicated that IL-3-induced blast colonies consisted of multipotential hematopoietic progenitor cells. By culturing individual IL-3-induced blast colonies in the presence of rIL-2 and irradiated peritoneal macrophages, on the other hand, the proliferation of homogenous lymphoid cells was observed in 5 of 24 wells, each of which received a single blast colony. Morphologically, they were typical large granular lymphocytes (LGL), and thus it was indicated that LGL could be differentiated directly from the hematopoietic progenitor cells utterly in vitro by rIL-2 and accessory macrophages. From one of these culture wells, a continuous LGL line, IL3B1, was successfully obtained. The proliferation of IL3B1 was dependent on IL-2 in the presence of accessory macrophages, but they no longer responded to IL-3, nor to another T cell growth factor, IL-4. Flowcytometric analysis indicated that the phenotype of IL3B1 was Thy-$1^+$,$T3^+$,$L3T4^-$,Lyt-$2^-$,$T200^+$Asialo $GM1^+$, whereas that of original IL-3-induced blast cells was Thy-$1^+$,$T3^-$,$L3T4^-$,Lyt-$2^-$,$B220^-$. The results suggested that the expression of T3 molecules was induced in the process of LGL differentiation from the hematopoietic progenitor cells in vitro. Conforming to this, it was revealed that both $\gamma$ and $\beta$ chain genes of the TCR were rearranged in IL3B1. Northern blot analysis indicated that IL3B1 had abundant mRNA for $\gamma$ chain, while mRNA for $\beta$ chain was rather faint. Functionally, IL3B1 exhibited typical NK-patterned cytotoxic activity against a panel of tumor cell targets. In addition, they showed significant cytotoxic activity against normal bone marrow cells, as well as various factor-dependent myelogenous progenitor cell lines, all of which were resistant to endogenous NK activity of the fresh spleen cells. These results indicated that at least a set of $T3^+$ LGL with rearranged TCR genes could be directly differentiated from isolated hematopoietic progenitor cells in vitro. Results also suggested that such a prethymically differentiated subset of T-lineage lymphocytes, namely $T3^+$ double-negative LGL, had particular cytotoxic activity in addition to conventional NK activity, which might well contribute to feedback regulation of hematopoiesis.

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