REGULATION OF THE GROWTH AND FUNCTIONS OF CLONED MURINE LARGE GRANULAR LYMPHOCYTE LINES BY RESIDENT MACROPHAGES

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Natural killer (NK)[1] cells represent a distinct subpopulation of normal lymphoid cells that has a characteristic morphology, termed large granular lymphocytes (LGL) (1). Despite accumulating evidence that NK cells play significant roles in certain aspects of host defense mechanisms (2), they largely remain enigmatic cells.

In terms of proliferation, for instance, the only clue has been that interleukin 2 (IL-2) could act on endogenous NK cells (3) and initiate their proliferation (3, 4). Unlike T cells, NK cells were shown to respond to IL-2 in normal animals without prior stimulations (5). Indeed, a number of continuous cell lines with NK-like activity have been established from normal lymphoid tissues using a variety of IL-2-containing conditioned media (CM) without particular antigens, in both man and mice (6–10). Subsequently, however, it was reported by Olabuenaga et al. (11) that IL-2 was a necessary but not sufficient factor to maintain the sustained proliferation of such cell lines with NK activity, although another cofactor(s) remained unelucidated. These results suggest that the IL-2-initiated proliferative response of endogenous NK cells is under regulation distinct from that of most T cells, where antigens in association with accessory cells have a primary role (12–15).

Functional aspects of the LGL-NK effector system are somewhat more complicated and confusing. Originally, NK cells have been reported as naturally occurring cytotoxic cells against certain malignant cells (16, 17) as well as various virus-infected cells (18, 19), but they have also been found to be cytotoxic to some normal cellular components, including normal lymphohematopoietic cells

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[1] *Abbreviations used in this paper:* $AsGM_1$, asialo GM_1; BMC, bone marrow cells; CFU-C, colony-forming units of committed progenitor cells; CM, conditioned medium; CSF, colony-stimulating factor; E/T, effector/target; FcR, Fc receptor; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HeLa-HSV, HeLa cells acutely infected with HSV; HeLa-Ms, HeLa cells persistently infected with measles virus; HSV, herpes simplex virus; IL-1, IL-2, interleukin 1 and 2; IL-2R, interleukin 2 receptor; LGL, large granular lymphocyte; LPS, lipopolysaccharide; NK cell, natural killer cell; PBS, phosphate-buffered saline; PEC, peritoneal exudate cells; ppIL-2, partially purified IL-2; PWM, pokeweed mitogen; rIL-2, recombinant IL-2; TdT, terminal deoxynucleotid transferase.

(20, 21) and fetal fibroblasts (1). Reflecting the latter activity, several reports indicated that purified NK cells could indeed suppress normal hematopoiesis as well as antibody responses in vitro (7, 22, 23). Furthermore, there is evidence suggesting that such reactivities to both normal and abnormal cells are mediated by the same set of effectors (22, 24), although it is not clear whether both activities are mediated by the same mechanism or not. However, it does not seem to make much sense that such basically contradictory reactivities are simultaneously triggered to operate in response to foreign cells, and would even be harmful to the host, unless they are well regulated in vivo.

We have established a number of cloned continuous murine LGL lines (25). Such LGL clones showed essentially the same phenotypical as well as functional characteristics as normal endogenous NK cells, and thus were considered endogenous NK cells rather than various anomalous killer cells generated in a variety of culture conditions (26, 27). Using such LGL clones, we studied at the clonal level the regulation of proliferation and of the functions of NK cells, as well as their mechanisms.

Materials and Methods

Mice. Female BALB/c and C57BL/6 mice were purchased from Shizuoka Experimental Animal Center, Japan.

Cell Lines. YAC-1 (A/J, Moloney-induced leukemia), RL♂1 (BALB/c, radiation-induced leukemia), EL4 (C57BL/6, thymoma), P815 (DBA/2, mastocytoma), LSTRA (BALB/c, Moloney-induced leukemia), MBL-2 (C57BL/6, Moloney-induced leukemia), and BW5147 (AKR, thymoma) cell lines were all cultured in suspension forms in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM Hepes, and Eagle's nonessential amino acids (complete RPMI). HeLa and BALB/c 3T3 were maintained in Eagle's minimum essential medium (MEM) supplemented with 5% FCS, glutamine, and antibiotics. The CTLL-2 line was maintained in complete RPMI 1640 supplemented with 20% rat concanavalin A (Con A)-conditioned medium (CM).

Virus-infected Cells. HeLa cells persistently infected with measles virus (HeLa-Ms) were originally established by Dr. J. Holland (University of California, San Diego) (28), and were maintained in our laboratory. Persistence of virus was routinely monitored by indirect immunofluorescence using both rabbit antimeasles serum and monoclonal antimeasles hemagglutinin antibody (H-1) (29). Nearly 100% of cells showed positive measles hemagglutinin expression. Herpes simplex virus (type I)–infected HeLa cells (HeLa-HSV) were made by acute infection for each experiment. Monolayers of HeLa were infected with HSV type I (Miyama strain) at 10:1 multiplicity of infection (moi) for 2 h. Over 90% of such treated cells showed positive HSV antigens on the surface as judged by immunofluorescence using rabbit anti-HSV serum.

Peritoneal Resident Macrophages. Normal peritoneal cells were harvested from aged mice by repeatedly rinsing peritoneal cavities with heparinized Hanks' solution. After washing, they were cultured overnight in complete RPMI medium in dishes (Nunc, Roskilde, Denmark). Culture dishes were thoroughly rinsed to eliminate nonadherent cells, and then irradiated with 3,000 rad γ radiation using a Gamma Cell (^{137}Cs; Atomic Energy of Canada, Ltd., Ottawa, Canada) before use as accessory cells.

LGL Lines and the Cloning. An IL-2-dependent, continuous LGL line was established from peritoneal exudate cells (PEC) of BALB/c mice injected intraperitoneally with 10^6 syngeneic "NK-sensitive" RL♂1 cells, which were not tumorigenic in these mice (25). Briefly, PEC were cultured at 10^6 cells/ml in complete RPMI in T-75 flasks (Corning Glass Works, Corning, NY) in the presence or absence of rat IL-2 that had been partially purified (pp) from Con A CM by DEAE cellulose and Sephadex G75 gel chromatography. The former cultures were fed with ppIL-2 every other day (10 U/ml). In 3 wk, the

outgrowth of floating cells became apparent in ppIL-2–fed cultures, whereas all the cells died out in cultures of medium alone. Such growing cells could now be propagated in the presence of rat ppIL-2 or human recombinant IL-2 (rIL-2) as well as irradiated peritoneal macrophages (IL-2-dependent PEC-1 line). Similar cell lines could be obtained from PEC of mice injected with virus-infected HeLa cells (PEC-2). Although unstimulated normal PEC showed undetectable levels of cytotoxic activity, PEC stimulated in vivo with either RLδ1 or virus-infected HeLa 3–5 d before were found to exhibit potent cytotoxic activity. Furthermore, the cytotoxic spectrum of these PEC was exactly parallel to that of endogenous NK activity and we observed no anomalous killer activity, which is often associated with various in vitro manipulated lymphoid populations. It thus seemed that such stimulated PEC were an ideal population from which to obtain normal NK cell lines.

Two major technical points were noted in establishing cell lines with IL-2 alone without any antigenic stimulation from stimulated PEC, the reason becoming evident later. First, the nonadherent lymphoid population should not be separated from adherent cells to start with; second, the original culture flasks, which were well coated with adherent cells, should not be changed for at least a few weeks until sufficient numbers of growing cells became evident. Otherwise, successful establishment of cell lines could not be achieved. Examination of the PEC-1 and PEC-2 lines indicated that the majority of cells had morphology typical of LGL.

Cloning was performed by limiting dilution procedures using flat-bottom microtiter plates (Costar, Data Packaging, Cambridge, MA). Each well received 10^5 normal peritoneal resident cells (0.1 ml) irradiated with 3,000 rad γ radiation, and then appropriately diluted PEC-1 cells (0.5 cell/0.1 ml) were added to each well. They were fed with 10 U/ml of human rIL-2 every other day. Based on an average of five independent experiments, the development of growing colonies was observed in 23% of total wells (23/96, 24/96, 13/96, 33/96, and 19/96). Cells from what appeared to be single colonies per well were transferred to 24-well plates (Costar) for expansion in the presence of irradiated peritoneal adherent cells. At this stage, developed clones were screened morphologically by Giemsa stain, and those with typical LGL morphology were selected and further expanded.

Cell Freezing. Cloned 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 only in the presence of irradiated peritoneal adherent cells.

IL-2 and IL-2 Assay. In most experiments, we used human rIL-2, originally produced at Biogen SA, Geneva, Switzerland and supplied by Shionogi & Co., Ltd., Osaka, Japan. Its specific activity was 10^8 U/mg protein and it contained 0.1% human serum albumin as a stabilizer. The IL-2 assay was performed using the CTLL-2 cell line.

Macrophage CM and IL-1 Assay. IL-1-containing macrophage CM was prepared as described by Lugar et al. (30). Briefly, normal human peripheral monocytes were cultured at 10^6 cells/ml in complete RPMI with 1% FCS in the presence of 100 μg/ml of silica for 2 d. Culture supernatant was harvested, centrifuged, and 15-fold concentrated by vacuum dialysis. The concentrated preparation contained 1,500 U/ml of IL-1 activity, but IL-2 activity was undetectable. From this, a partially purified IL-1 fraction was obtained by AcA54 Ultrogel (Villeneuve la Garenne, France) chromatography (13–17,000 mol wt). The IL-1 assay was performed by a thymocyte costimulating assay, using 1 μg/ml of Con A.

Cellular ^{125}I-labeled IL-2 Binding Assay. IL-2 was radioiodinated using ^{125}I-labeled Bolton and Hunter's reagents (2,000 Ci/mmol, monoiodinated, NEX-120; New England Nuclear, Boston, MA) (31). 10 μg of rIL-2 was dissolved in 10 μl of 0.2 M borate buffer (pH 8.5) and incubated with dried 100 μCi of the reagent in a 1.5 ml Eppendorf microtube at 0°C for 30 min. After addition of 50 μl 0.5 M glycine/0.1 M borate buffer, the mixture was incubated for an additional 15 min, and then fractionated through a glass column (0.7 × 25 cm) packed with Sephadex G-50 equilibrated with 0.05 M sodium phosphate buffer (pH 7.5) containing 0.5% (wt/vol) bovine serum albumin. The specific radioactivity was 2,000 cpm/ng.

To determine the level of binding, cells were extensively washed (usually four times)

with cold complete RPMI with 10% FCS to remove bound IL-2, and then adjusted to 10^7 cells/ml. A series of twofold dilutions of ^{125}I-IL-2 in a 0.1 ml vol was made, starting at 20 ng ^{125}I-IL-2 in Eppendorf microtubes; 0.1 ml of cell suspension (10^6 cells) was added to each tube. In one tube, 500 times excess cold IL-2 (10 μg) was added in addition to the ^{125}I-IL-2, to provide a basis for nonspecific binding. Tubes were then incubated in a 37°C water bath for 30 min. After incubation, 1 ml cold medium was added to each tube to stop the reaction, and the tubes were vortexed and centrifuged using a microfuge (9,000 rpm, 15 s). 1 ml supernate was removed from each tube for later counting and the rest of the media-containing cell pellets were resuspended. They were then centrifuged (5,000 rpm, 2 min) through a 0.2 ml layer of a mixture of 84% silicone oil and 16% paraffin oil. The tips of tubes containing the cell pellets were cut off and placed in γ-counting tubes for the determination of cell-bound IL-2. The remaining portions of the tubes were pooled with the corresponding first 1-ml supernatants and counted for free IL-2. Radioactivity was counted on a γ counter (Aloka Co. Ltd., Tokyo).

Antibodies and Flow Cytometry. Monoclonal anti-Thy-1.2 (IgM), anti-Lyt-1.2 (IgG2b), and anti-Lyt-2.2 (IgM) were purchased from New England Nuclear. Monoclonal anti-T200 (IgG1) and rabbit anti-asialo GM_1 ($AsGM_1$) were kind gifts from Dr. B. Bloom (Albert Einstein College of Medicine) and Dr. M. Kasai (National Institutes of Health, Japan), respectively. Monoclonal anti-mouse IL-2 receptor (IL-2R) antibody (AMT-13) has been described (32). Cells were washed three times in RPMI 1640, adjusted to 10^6/ml, and incubated with appropriate dilutions of antibodies for 30 min at 4°C. After incubation, they were washed three times, and then incubated with either fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG, rabbit anti-mouse IgM [$F(ab')_2$], or goat anti-rabbit IgG for another 30 min. After washing, fluorescence intensities were analyzed by Spectrum III (Ortho Pharmaceutical, Raritan, NJ).

Cytotoxicity Assay. For the radiolabeling of leukemic targets and normal bone marrow cells (BMC), 0.1 ml cells (10^6 and 10^7 cells, respectively) were incubated with 50 and 200 μCi of ^{51}Cr (New England Nuclear) for 2 h, respectively. HeLa-HSV, HeLa-Ms, and uninfected HeLa were similarly labeled with 50 μCi ^{51}Cr after trypsinization. Various dilutions of effector cells (0.1 ml) were mixed with 10^4 labeled leukemia or BMC targets in round-bottom microtiter wells (Linbro Scientific Co., Hamden, CT) or with 5×10^3 of labeled infected or uninfected HeLa cells in flat-bottom microtiter wells (Costar). The assay time was 5 h for the former targets and 18 h for the latter. These times were chosen on the basis of the kinetics of cytotoxicity of normal spleen cells against these targets (33). Supernatants were harvested by a semiautomatic harvesting system (Skatron AS, Lier, Norway) and counted on a γ counter (Aloka Co. Ltd.). The calculation of specific ^{51}Cr release has been described (33). Spontaneous release averaged 10% for leukemic targets, 25% for BMC, and 25% for virus-infected or uninfected HeLa cells.

Glutaraldehyde Treatment. Target cells used as cold inhibitors in cold-target inhibition experiments of cytotoxicity were all fixed with 0.05% glutaraldehyde in phosphate-buffered saline (PBS) to eliminate interactions between hot and cold targets. Cells were suspended in 0.05% glutaraldehyde in PBS, incubated at 37°C for 1 h, and washed four times before use. Preliminary experiments indicated that 0.05% was the maximum concentration of glutaraldehyde to be used without affecting the cold-competing capacity of target cells.

CFU-C Assay. The assay for colony-forming units of committed progenitor cells (CFU-C) was performed using a single-layer agar culture method. Normal BALB/c BMC were depleted of phagocytic macrophages by the carbonyl iron method. 5×10^4 of such BMC, preincubated with or without LGL clones for 2 h, were mixed in 1 ml of Iscove's modified Dulbecco's medium (Gibco Laboratories, Grand Island, NY) containing 0.3% agar (Difco Laboratories, Inc., Detroit, MI), 20% FCS, and 20% standard colony-stimulating factor (CSF). 5-d culture supernatant of spleen cells stimulated with pokeweed mitogen (PWM) was used as a source of standard CSF. A 1 ml aliquot was plated on a 35 mm dish (Falcon Labware, Oxnard, CA). After 7 d incubation, colonies of 50 or more cells were scored microscopically.

Results

Continuous Growth of Cloned LGL Lines Requires rIL-2 and Either Macrophages or Activated Macrophage-derived Factor(s). The PEC-1 line as well as all of the cloned LGL lines thereof displayed the serological phenotypes Thy-1^+, Lyt-1^-2^-, $AsGM_1^+$, $T200^+$, terminal deoxynucleotid transferase $(TdT)^-$, and Fc receptor $(FcR)^+$, without exception (25). As is shown in Fig. 1*A*, rIL-2 (0.1–100 U/ml) could initiate the proliferation of the clone, but the effect was only transient and the cells went into crisis despite repeated feedings with rIL-2. In contrast, when the cloned cells were cultured in the presence of irradiated normal peritoneal macrophages and similarly fed with rIL-2, they showed sustained proliferation with as little as 0.1 U/ml rIL-2 (Fig. 1*B*). Macrophages alone without rIL-2 induced little proliferation. In Fig. 2, growth patterns of two different states of clone are indicated: cells directly recovered from the culture with both rIL-2 and macrophages (Fig. 2*A*) and those similarly recovered but precultured with rIL-2 alone for 3 more days (Fig. 2*B*). These cells were adjusted in cell number (5×10^4/ml) and cultured in various culture conditions. In the latter case, even a transient proliferative response could not be obtained with rIL-2 alone, and a lag phase of a few days was required before cell growth became apparent in the presence of both rIL-2 and syngeneic macrophages. The cells apparently lost the proliferative responsiveness to rIL-2 in the absence of macrophages, but the

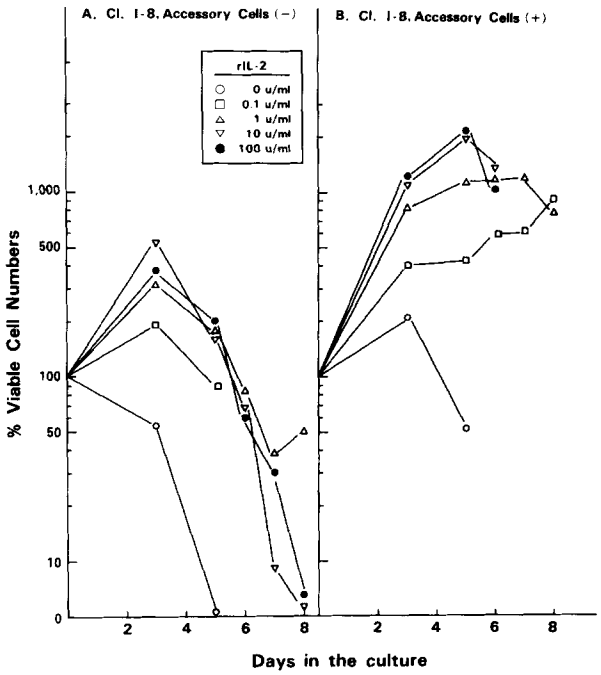

FIGURE 1. Proliferative response of a cloned LGL line (Cl.1-8) to rIL-2 in the absence (*A*) or presence (*B*) of macrophages. 10^5 Cl.1-8 were cultured in 2 ml of complete medium containing none or various doses of rIL-2 (0.1–100 U/ml) in the absence or presence of 10^6 of 6,000-rad-irradiated resident peritoneal adherent cells in 12-well dishes. In both cases, half of the medium (1 ml) of each well was replaced with new medium and fed with the indicated doses of rIL-2 every other day.

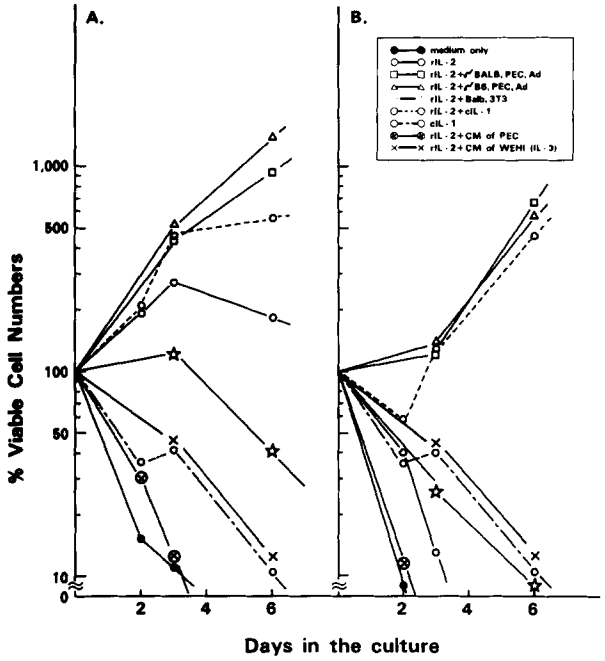

FIGURE 2. Effects of various cells and factors on IL-2-induced proliferation of an LGL clone. Cells of clone 1C were immediately harvested (*A*) from culture supplemented with rIL-2 (10 U/ml) and irradiated adherent cells or (*B*) after being precultured with rIL-2 (10 U/ml) alone for 3 d. 10^5 of these cells were then cultured with 10 U/ml of rIL-2 combined with various adherent cells (irradiated BALB/c or C57BL/6 peritoneal adherent cells, or 3T3) or with cell-free culture supernatants (cIL-1, crude CM of silica-stimulated human macrophages containing IL-1; or plain CM of normal peritoneal adherent cells containing no IL-1 activity). All these cultures were fed with 10 U/ml of rIL-2 every other day.

responsiveness could be regained upon reculture with macrophages. Allogeneic (B6) irradiated macrophages (Fig. 2) were as effective as syngeneic ones in supporting the growth of LGL clones, whereas 3T3 (fibroblast) cells were without effect. Although not shown, MG3T3 reticulum cells were by far less effective than macrophages. On the other hand, IL-1-containing CM of silica-stimulated human monocytes could effectively replace macrophages (Fig. 2). Since the partially purified (pp) IL-1 fraction by Ultrogel of the CM also supported the growth when combined with rIL-2, it seems likely that the active factor is IL-1. In contrast, plain CM of irradiated macrophages did not contain detectable level of IL-1 activity and could not replace accessory cells (Fig. 2). IL-3-containing WEHI CM, which supported the growth of bone marrow–derived mast cell lines, was also ineffective in replacing macrophages. Thus, such clones could be continuously propagated in culture only by using either macrophages or ppIL-1 from CM of stimulated macrophages in addition to rIL-2 (Fig. 3, *A* and *B*).

Macrophages Affect IL-2 Receptor Expression on Cloned LGL Lines. Since the primary growth factor for LGL was apparently IL-2, we presumed that the growth-supportive role of macrophages might be due either to their inducing endogenous IL-2 production of clones or to their affecting the receptors for IL-2 (IL-2R) on the clones. The first possibility seemed unlikely, since an excess

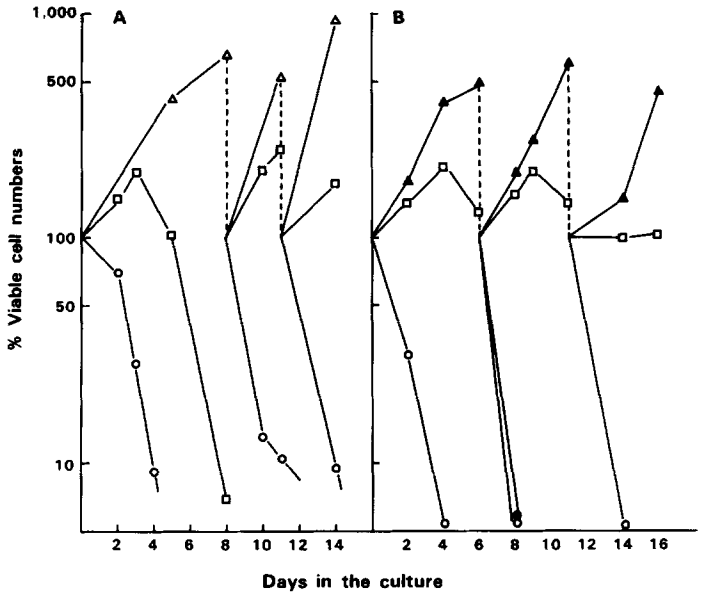

FIGURE 3. Continuous propagation in vitro of LGL clones by rIL-2 and normal peritoneal macrophages (*A*) or by rIL-2 and ppIL-1 (*B*). (*A*) Clone I-8 (10^5) were cultured alone (○), with 10 U/ml of rIL-2 (□), or with rIL-2 (10 U/ml) and 10^6 irradiated BALB/c peritoneal adherent cells (△). (*B*) Clone I-8 (10^5) were cultured alone (○), with 10 U/ml of rIL-2 (□), with 50 U/ml ppIL-1 (●), or with rIL-2 and ppIL-1 (▲).

amount of IL-2 had been consistently supplied exogenously into the system, and also no evidence of IL-2 production by clones was obtained even with various stimuli (data not shown). The second possibility was then examined, first using monoclonal anti-mouse IL-2R antibody. As shown in Fig. 4, approximately half of the cloned cells showed positive IL-2R expression as judged by flow cytometry. Almost all cells of CTLL-2 line expressed IL-2R, whereas the IL-2-independent T cell line (2E10.4.13) (34) expressed no detectable IL-2R. However, when IL-2R expression was compared between the LGL clone (Cl.I-8) cultured with rIL-2 alone (Fig. 4 *C*) and that cultured with both rIL-2 and macrophages (Fig. 4 *D*), no significant difference was observed in the degree of overall IL-2R expression. Similar results were obtained using a ^{125}I-IL-2 binding assay. As shown in Table I, the average number of IL-2-binding sites per cell was ~60–70 × 10^3 in various LGL clones, but no significant differences in total binding sites were observed between the populations maintained in the presence or absence of macrophages in any of LGL clones.

Thus, we considered the alternative possibility that macrophages affected the nature, rather than the number, of IL-2R in the overall populations of clones. For this, Scatchard plot analysis of cellular IL-2 binding was performed using ^{125}I-labeled rIL-2 (Fig. 5). When Cl.I-8 was cultured for 5 d with both rIL-2 and macrophages, the presence of IL-2R with distinct affinities was evident: high affinity binding sites of dissociation constant (K_d) ~0.5 nM (average 12 × 10^3 sites per cell) and low affinity sites of K_d >10 nM (Fig. 5*A*). In contrast, Cl.I-8 cultured for 5 d with rIL-2 in the absence of macrophages showed only low affinity IL-2R, and no apparent high affinity IL-2R. Essentially similar results

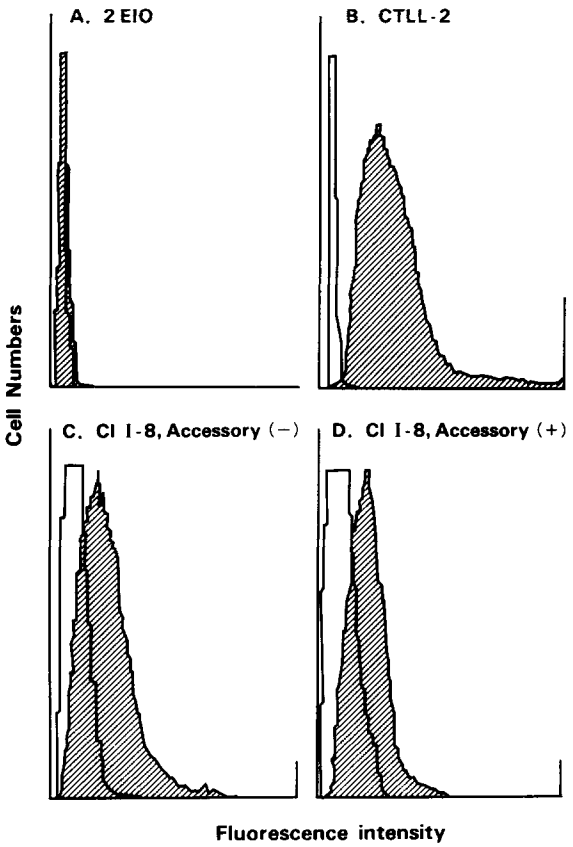

FIGURE 4. Flow cytometry analysis of IL-2R expression of LGL clone by monoclonal anti-IL-2R antibody. Factor-independent T cell line, 2E10 (*A*), IL-2-dependent T cell line, CTLL-2 (*B*), or LGL clone, I-8, either cultured with rIL-2 alone for 5 d (*C*) or with rIL-2 and irradiated peritoneal adherent cells for 5 d (*D*), were washed three times in IL-2-free cold medium. 10^6 of these cells were incubated with medium (□) or with monoclonal anti-IL-2R antibody (AMT-13) (▨) for 30 min at 4°C, and washed twice. They were then incubated with FITC-conjugated rabbit anti-mouse IgG [$F(ab')_2$] for 30 min. After washings, fluorescence intensities of these cells were analyzed using Spectrum III (Ortho Pharmaceutical).

were obtained using another LGL clone, 1C (Fig. 5*B*). When Cl.1C was cultured with rIL-2 and macrophages, the presence of high affinity IL-2R (K_d, 0.23 nM; 8×10^3 site per cell) and low affinity IL-2R, (K_d, 30 nM) was evident. In contrast, when the clone was cultured with rIL-2 alone, the number of high affinity IL-2R was clearly reduced, whereas low affinity IL-2R were nearly as abundant as when cultured with rIL-2 and macrophages. The results thus indicate that macrophages increase the degree of high affinity IL-2R expression in the overall population of cloned LGL without significantly affecting the total number of IL-2R.

Characteristics of Cytotoxicity of Cloned LGL Lines. Using cloned LGL lines that now could be stably propagated in culture, we systematically examined the cytotoxic activity. It should be noted that the clones were randomly selected on the basis of morphology (LGL), not on the basis of cytotoxic activity against any

TABLE I

Cellular ^{125}I-IL-2 Binding on Cloned LGL Lines Cultured in the Presence or Absence of Resident Macrophages

Clones	PEC in culture	Cold IL-2*	Bound IL-2 per 10^6 cells	Specific binding per 10^6 cells	Average binding sites per cell
			cpm	*cpm*	
C1.I-8	−	−	3,305	2,947	58×10^3
		+	358		
	+	−	4,311	3,841	76×10^3
		+	470		
C1.1H5	−	−	2,531	2,314	63×10^3
		+	217		
	+	−	2,776	2,593	70×10^3
		+	183		
C1.I-5	−	−	2,930	2,713	73×10^3
		+	217		
	+	−	2,940	2,751	74×10^3
		+	189		

Cloned LGL lines were cultured with 50 U/ml of rIL-2 in the presence or absence of 3,000-rad–irradiated macrophages for 3–5 d. Cells were then harvested and washed, and an IL-2 binding assay was performed as described in Materials and Methods. Specific activity of ^{125}I-IL-2 was 1,980 cpm/ng for the first experiment, and 1,445 cpm/ng for the last two experiments.

* One million cells of cloned lines were aliquoted into each of two tubes. In one tube, 9 ng of ^{125}I-IL-2 was added, and in the other tube, 5 μg of cold IL-2 (555 times excess) was added simultaneously with 9 ng of ^{125}I-IL-2.

particular target. As targets, three major categories of cells were used, leukemic cells, including both NK-sensitive and -resistant lines (group I), HeLa cells infected with viruses (measles, HSV) as well as an uninfected control (group II), and normal fresh bone marrow cells (BMC) (group III). The representative cytotoxic patterns of cloned LGL lines at various effector/target (E/T) ratios, as well as the summary of repeated experiments, are indicated in Fig. 6 and Table II, respectively. Both uncloned parental lines, PEC-1 and PEC-2, established in independent experiments from different animals, exhibited essentially the same cytotoxic spectrum as endogenous NK cells in normal spleen. We observed no cytotoxic activity such as anomalous killer (AK) activity, which is usually generated only by various in vitro manipulations. However, the cytotoxic spectra of individual clones derived from the PEC-1 line varied significantly. None of the clones affected the target cells p815, EL4, LSTRA, BW5147, MBL-2, and HeLa, which normal splenic NK cells could not kill. Another notable feature was that almost all the randomly cloned LGL lines showed various degrees of cytotoxic activity against normal fresh BMC and virus-infected HeLa, whereas only selective clones killed leukemic cells.

Although lines used here had been cloned under stringent conditions, the possibility existed that the lines were still mixtures of clones with distinct specificities. To confirm the clonality of lines, we performed cold-target inhibition experiments among different groups of targets (Fig. 7). Since large numbers of

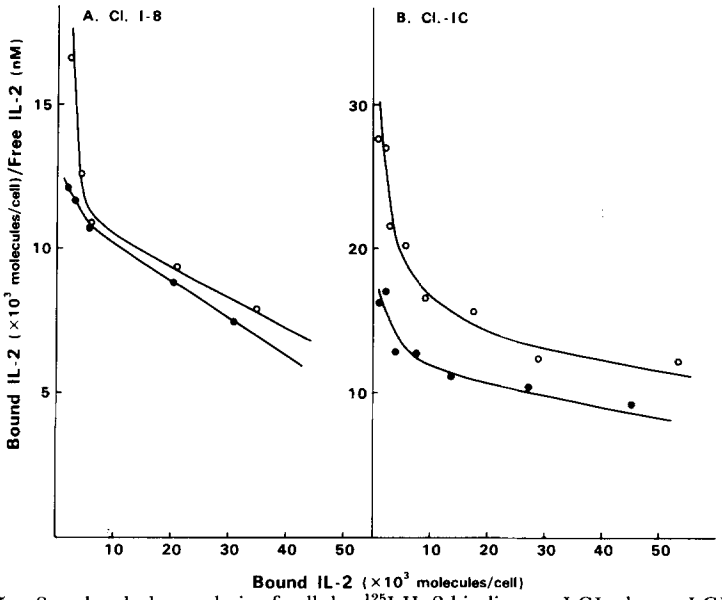

FIGURE 5. Scatchard plot analysis of cellular ^{125}I-IL-2 binding on LGL clones. LGL clones, I-8 (*A*) and 1C (*B*), were cultured with 10 U/ml of rIL-2 in the presence (○) or absence (●) of irradiated BALB/c peritoneal adherent cells for 5 d. Nonadherent cells were then harvested, washed three times in cold IL-2-free medium, and resuspended at 10^7 cells/ml. The IL-2 binding assay was performed as described in Materials and Methods.

leukemic cells used as cold inhibitors often by themselves affected labeled targets, especially BMC, those used as cold targets were pretreated with 0.05% glutaraldehyde to eliminate such effects. The cytotoxic activity of Cl.1H5 against RLδ1 was significantly inhibited by homologous RLδ1 as well as by HeLa-Ms cells, whereas cold EL4 hardly affected it (Fig. 7*B*). Rather unexpectedly, cold YAC-1, which was not affected by the particular clone, also inhibited Cl.1H5 although to a less striking degree compared with normal splenic NK cells (Fig. 7*A*). As shown Fig. 7*C*, cytotoxic activity of 1H5 against labeled BMC was inhibited by syngeneic and allogeneic BMC but also, and more strikingly, by leukemic cells (RLδ1 and, less efficiently by YAC-1). Cold-competing activity was also compared among various lymphoid cell components, using a much lower E/T ratio (0.1:1) to make inhibition easier. Con A- and lipopolysaccharide (LPS)-induced blast cells competed for normal BMC as efficiently as by homologous BMC targets, whereas normal splenocytes and thymocytes competed far less effectively (Fig. 7*D*). Nontheless, Cl.1H5 displayed only marginal direct cytotoxic activity against Con A and LPS blasts (data not shown).

The results confirmed that a single LGL clone could affect varied combinations of three representative groups of NK targets, and also suggested that the binding of LGL clones to certain targets might not necessarily result in apparent cytolysis.

Effect of Macrophages on Functional Expression of LGL Clones. Our next question was whether accessory macrophages could influence functional expression of LGL clones in addition to proliferation. LGL clones were cultured with rIL-2 in the presence or absence of syngeneic macrophages for 2 d, and nonadherent LGL were harvested and assayed for cytotoxic activity against three groups of

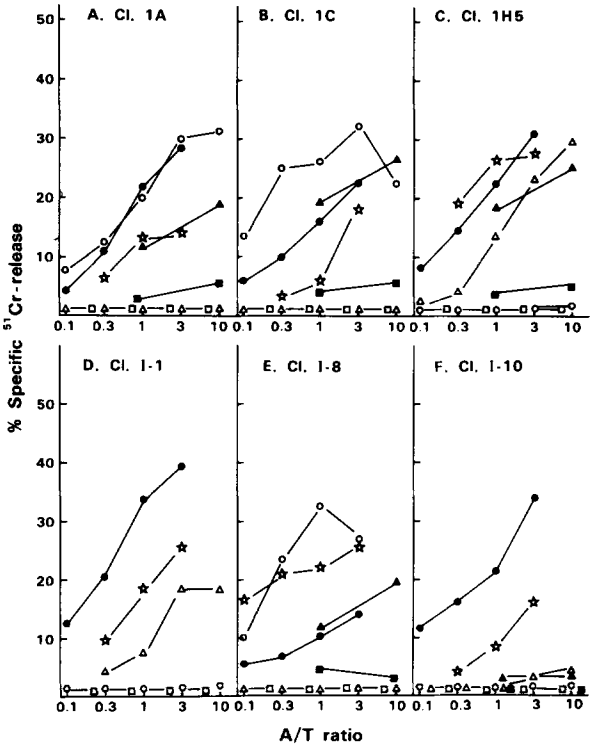

FIGURE 6. Cytotoxic spectra of cloned LGL lines at various E/T ratios. Target cells used were as follows. Group I: RLδ1 (△), YAC-1 (○), and EL4 (□); group II: uninfected HeLa (■), HeLa persistently infected with measles virus (●), and HeLa acutely infected with HSV type 1 (▲); and group III: normal BALB/c BMC (☆). Assay times were 5 h for group I and III targets, and 18 h for group II targets. Spontaneous releases were 8–12%, 20–25%, and 20–30%, respectively, for group I, II, and III targets.

target cells. Results are summarized in Table III. When leukemia cells (RLδ1) or virus-infected cells (HeLa-Ms) were used as targets, Cl.1H5 maintained with macrophages showed significantly higher cytotoxicity than those without. In contrast, the cytotoxic activity against normal BMC was rather depressed when the clone was cultured with macrophages. Such opposite effects of resident macrophages on the cytotoxic activity of a single clone against normal and malignant or virus-infected target cells was also observed using other LGL clones (Table III). However, macrophages did not affect the cytotoxic spectrum of the LGL clones per se. For instance, macrophages augmented the cytotoxic potential of Cl.1H5 against RLδ1, but could not induce cytotoxic activity against YAC-1, which the clone originally could not kill. The same was true for Cl.IV-9 against RLδ1 (Table III). Thus, although LGL clones were potentially cytotoxic to part of normal BMC, the presence of macrophages induced a shift of their cytotoxic patterns toward malignant or virus-infected cells.

Effect of LGL Clones on In Vitro Hematopoiesis and its Regulation by Macrophages. Finally, to examine the physiological significance of cytotoxic activity of LGL against normal BMC and its negative regulation by macrophages, we investigated the effect of LGL clones on in vitro hematopoiesis. Various numbers

TABLE II
Cytotoxic Spectra of Cloned Continuous LGL Lines

Effectors:	Group I (leukemias)					Group II (virus)			Group III (normal BMC)	
	YAC-1	RLδ1	P815	EL4	MBL-2	HeLa	HeLa-MS	HeLa-HSV	BALB/c	C57BL/6
Normal spleen: cells	++*	++	−	−	−	−	++	+	+/−	+/−
PEC-1	++	++	−	−	−	−	++	ND‡	++	++
Cl.1C	++	−	−	−	−	−	+	++	+	ND
Cl.1A	++	−	−	−	−	−	++	+	+	ND
Cl.1H5	−	++	−	−	−	−	++	++	++	++
Cl.I-1	−	+/−	−	−	−	−	++	+/−	++	ND
Cl.I-5	++	−	−	−	−	−	++	ND	++	++
Cl.I-6	−	−	−	−	−	−	−	ND	+	ND
Cl.I-8	++	−	−	−	−	−	+	++	++	++
Cl.I-10	+/−	+/−	−	−	−	−	++	−	+	ND
Cl.IV-4	−	−	−	−	−	−	ND	ND	ND	ND
Cl.IV-9	−	−	−	−	−	−	+	ND	+	ND
PEC-2	++	++	−	−	ND	ND	+	ND	+	+

Cytotoxic activity of normal BALB/c spleen cells as well as PEC-1 line, PEC-2 line, and the LGL clones thereof was assayed against a number of target cells using a ^{51}Cr release assay. PEC-1 and PEC-2 lines were obtained independently from different animals, using RLδ1 as a booster for PEC-1 and HeLa-Ms for the PEC-2 line. Targets included NK-susceptible and -resistant malignant cell lines (group I), measles- or HSV-infected HeLa cells as well as uninfected HeLa (group II), and syngeneic or allogeneic normal BMC.

* Gradings of cytotoxic activity were as follows: (−) 0–5%, (+/−) 5–10%, (+) 10–20%, (++) >20%, at an E/T ratio of 1–3:1 for clones and 100:1 for normal spleen cells. The cytotoxic assay was performed two to six times for each clone, and the average values were scored.

‡ Not done.

of Cl.1H5, which had been cultured with rIL-2 either in the absence or presence of irradiated macrophages for 2 d, were mixed with 5×10^4 of normal BALB/c BMC depleted of phagocytic cells. The mixtures of cells were incubated at 37°C for 2 h. They were then inoculated into 0.3% agar including 20% PWM-stimulated spleen cell CM as a source of CSF, in 35-mm dishes. The number of granulocyte/macrophage (GM) colonies was counted after 7 d incubation by double staining. The representative results are shown in Fig. 8. Cl.1H5 cultured without macrophages exhibited significant inhibitory effect on the development of GM-CFU from normal BMC. The cloned cells cultured with macrophages, on the other hand, showed much less inhibitory effect than those without (Fig. 8*A*). Thus, the regulation of LGL clones by macrophages for the inhibitory potential of in vitro hematopoiesis was apparently parallel to that for the direct cytotoxic activity, as judged by short-term ^{51}Cr release (Fig. 8*B*). The IL-2-dependent CTLL-2 line as a control exhibited no inhibitory effect on GM-CFU development and only marginal cytotoxic activity against BMC.

Discussion

The first part of our study concerns the growth requirements for cloned LGL lines. The results indicated that the presence of both macrophages and IL-2 was

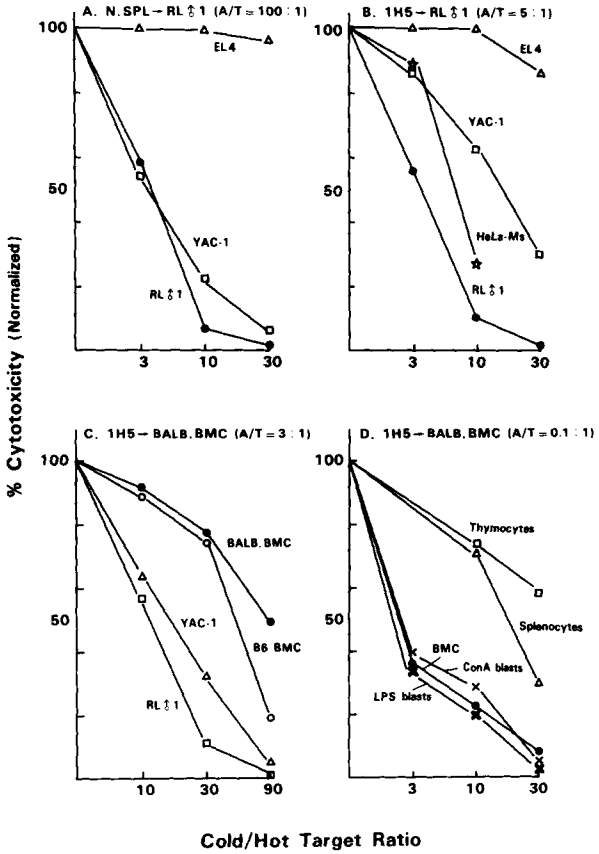

FIGURE 7. Cold-target inhibition experiments among various targets for normal (nylon wool purified) spleen cells (*A*) and for cloned LGL line, 1H5 (*B*, *C*, *D*). Target cells used as cold inhibitors were all pretreated with 0.05% glutaraldehyde for 1 h at 37°C, and washed four times before use. The percent specific ^{51}Cr release were as follows. (*A*) Normal nylon wool–purified BALB/c spleen cells against RLδ1 at a 100:1 E/T ratio, 37.5%; (*B*) clone 1H5 against RLδ1 at a 5:1 E/T ratio, 38.0%; (*C*) clone 1H5 against normal BALB/c BMC at a 3:1 E/T ratio, 32.0%; and (*D*) clone 1H5 against normal BALB/c BMC at a 0.1:1 E/T ratio, 18.7%. Assay time was 5 h.

a necessary and sufficient condition for sustained proliferation of the clones. Allogeneic macrophages supported the growth as efficiently as syngeneic ones. On the other hand, the 3T3 fibroblast line could not support the IL-2-dependent growth of LGL clones at all. The MG3T3 reticulum cell line, shown to be able to support the growth of hematopoietic stem cells (35), was also far less effective compared with macrophages (data not shown). These results suggested that the interaction of cloned LGL with macrophages, in terms of proliferation, was rather cell type specific. The role of macrophages was found to be replaced by partially purified IL-1, which is known to be primarily produced by the monocyte/macrophage lineage of cells, whereas IL-3-containing WEHI CM was without effect. Collaborative activity of IL-1 with IL-2 in stimulating NK activity has been reported (36). It thus seems probable that a variety of crude CM successfully used by others to maintain cell lines with NK-like activity contained IL-1-like

TABLE III

Differential Regulatory Effects of Normal Resident Macrophages on the Cytotoxic Potential of Cloned LGL Lines against Normal BMC and Leukemic or Virus-infected Target Cells

Clones	Target cells	No. of experiments	PEC in culture	Cytotoxicity* at E/T ratio of:			
				10	3	1	0.3
				%			
1H5	BMC	4	−	34.8	30.1	23.2	12.8
			+	8.9	9.9	9.2	2.3
	RLδ1	4	−	11.8	8.7	4.7	0.3
			+	25.5	15.9	6.2	0.4
	YAC-1	2	−	2.7	2.1	1.6	—
			+	1.4	1.2	1.0	—
	HeLa-Ms	2	−	22.7	21.9	19.2	10.5
			+	43.3	35.0	17.9	10.6
IV-9	BMC	1	−	37.9	39.8	29.4	25.6
			+	25.9	11.0	8.3	8.2
	RLδ1	1	−	0	0	0	0
			+	0	0	0	0
	HeLa-Ms	1	−	6.6	25.2	22.5	11.7
			+	27.9	43.8	28.5	12.2
I-A	BMC	1	−	39.5	40.9	30.3	—
			+	0	0.9	4.2	—
	HeLa-Ms	2	−	—	24.6	19.4	12.3
			+	—	37.3	27.4	10.5
I-8	BMC	1	−	34.6	26.7	24.7	17.8
			+	7.3	1.9	3.6	4.9
	HeLa-Ms	2	−	7.6	10.0	8.8	6.2
			+	19.0	21.4	17.4	10.4

5×10^5 cells of each of cloned LGL lines were cultured with 10 U/ml of rIL-2 in the absence or presence of 5×10^5 of 6,000-rad–irradiated peritoneal resident macrophages for 2 d. Nonadherent cells were then harvested, washed, and assayed for cytotoxic activity against various target cells using a ^{51}Cr release assay. There was negligible carryover of macrophages.

* Mean specific ^{51}Cr release of the indicated number of independent experiments is shown.

factor(s) in addition to IL-2. It remains to be seen whether the function of macrophages is mediated through soluble factor(s), since plain CM of resident macrophages contained undetectable levels of IL-1 activity and could not replace macrophages (Fig. 2). Indeed, we have preliminary evidence that intimate contact between LGL and macrophages is needed for optimal growth promotion. At present, it is fair to say that either macrophages or activated macrophage–

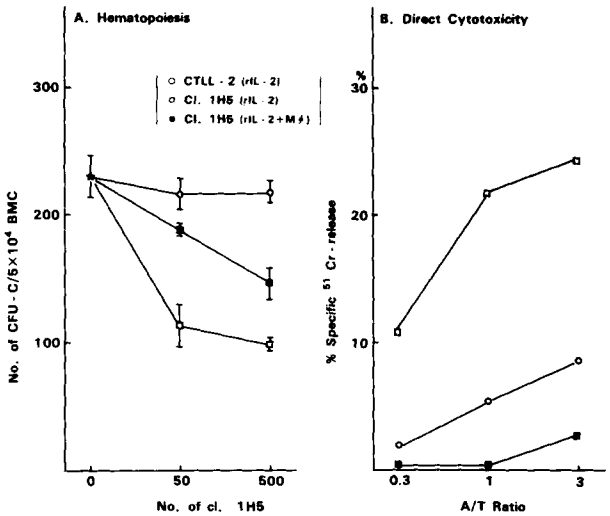

FIGURE 8. Suppression of in vitro hematopoiesis (*A*) as well as direct cytotoxicity against normal BMC (*B*) by cloned LGL. (*A*) Cl.1H5 either maintained with rIL-2 alone (□) or with rIL-2 in the presence of macrophages (■) for 2 d as well as IL-2-dependent CTLL-2 (○) as a control, were mixed with 5×10^4 of macrophage-depleted normal BALB/c BMC at various ratios and incubated for 2 h. They were then inoculated into 0.3% agar Iscove's Dulbecco's modified Eagle's medium containing CSF and cultured for 7 d. Plates were then double stained and GM colonies were counted. The mean numbers ± SD of CFU-C of triplicate dishes are indicated. (*B*) Aliquots of Cl.1H5 and CTLL-2 prepared as above were mixed with ^{51}Cr-labeled normal BMC at various ratios and specific ^{51}Cr release was determined by a 5 h assay.

derived CM containing IL-1 is required, in addition to IL-2, for the sustained proliferation of LGL clones, which may or may not involve common processes.

Several possibilities can be considered for the mechanisms of the macrophage dependency of LGL growth. For instance, macrophages may nutritionally support LGL, secreting factors such as glutathione (37). As indicated, however, plain CM of macrophages could not replace macrophages; moreover, 2-mercaptoethanol, known to substitute for such macrophage activity (38), did not affect the growth of the clones at all (data not shown). We thus presumed that the role of macrophages is a more specific one. Since it was evident that the primary growth factor for LGL was IL-2, our major effort was directed to the analysis of IL-2R expression on the clones. At a populational level, however, no significant difference in the degree of IL-2R expression was observed between LGL clones cultured with macrophages and those without, either by staining with anti-IL-2R antibody or by ^{125}I-IL-2 binding assay (Fig. 4, Table I). In contrast, a Scatchard plot analysis of ^{125}I-IL-2 binding indicated a significant difference between these two states of LGL clones. Clones maintained with macrophages showed distinct high affinity IL-2 binding sites, with approximate K_d of 0.2–0.5 nM (average, 10×10^3 sites per cell), whereas those without macrophages exhibited few or much reduced numbers of high affinity IL-2R (Fig. 5). On the other hand, LGL in both states expressed comparable numbers of lower affinity IL-2R (K_d, 10–30 nM). Since an average number of high affinity IL-2R was <20% of total IL-2R (60–70 × 10^3 sites per cell), it seems reasonable that such a difference could not be revealed by the analysis with anti-IL-2R antibody. The

presence of IL-2R with distinct affinities was recently demonstrated by Robb et al. (39), using a battery of conventional T cells. The affinities of IL-2R on the present LGL clones were ~10 times lower than those reported on conventional T cells (39). Since we detected comparable affinities of IL-2R on T cell lines by our method (40), we feel that the reduced affinities of IL-2R on LGL compared with T cell (lines) are attributable to the intrinsic features of this type of cells rather than to technical problems.

The mechanism by which macrophages affect IL-2R expression on cloned LGL is unclear at present, primarily because expression of IL-2R is apparently heterogeneous among cells, even though they were clonal, and the present analysis only applied to the populational level. Possible explanations may include (*a*) macrophages which selectively maintain cells with high affinity IL-2R, which in turn efficiently responds to IL-2, and (*b*) macrophages which actively induce high affinity IL-2R on LGL, which tend to be progressively lost with IL-2 alone in the absence of macrophages. We favor the last explanation since the phenomenon was a reversible one, but it remains to be proved. In any case, the finding that macrophages maintain high affinity IL-2R on LGL clones would explain the ready-made responsiveness of endogenous NK cells to IL-2, without exogenous stimulation unlike T cells. Recently (41), IL-1 was shown to be able to induce IL-2R on T cells. Thus, IL-1 may share with macrophages the mechanism for affecting LGL growth.

One of the most striking features of normal NK cells is a peculiar, distinct cytotoxic spectrum, including some transformed cells, especially of lymphoid origin, a variety of virus-infected cells, and some normal lymphohematopoietic cells. Such a characteristic preference for peculiar targets functionally distinguishes them from other nonspecific killer cells, such as spontaneous cytotoxic cells (42), anomolous killer cells (27), lymphokine-activated killer cells (43), and variously activated phagocytic cells (44). All of our present clones showed typical LGL morphology, characteristic phenotypic features without any T cell differentiation antigens, and, most importantly, the same typical spectrum of cytotoxic potential as that of endogenous NK cells. We thus believe that the present LGL clones represent definitive, endogenous NK cells rather than cells with anomolous killer activity that primarily appear in cultures.

Functional analysis of LGL clones indicated the following points. (*a*) A single LGL clone can kill multiple target cells that are completely unrelated. This is the first indication that cloned lines with typical NK cell features could affect virus-infected cells and normal BMC along with susceptible leukemia cells. The single clonal nature of such cytotoxicity was confirmed by cold inhibition experiments. The clones did not affect any of six other target cells that were resistant to normal NK-mediated cytotoxicity. (*b*) Cloned LGL lines, nevertheless, showed rather heterogeneous patterns of cytotoxicity within the original spectrum (Table II). A similar observation has been reported by Herzend et al. (45) in a human system, using a series of lymphoma targets. Based on this clonal heterogeneity, Herzend et al. (46) identified a clonotypic molecule on some selected NK clones, with a similar structure, as probable T cell receptors. We have evidence that T cell receptor β genes (C_β) of present LGL clones have all been rearranged from a germline structure (if not completely), as judged by Southern hybridization

analysis (Ikuta, Minato, Yodoi, and Honjo, manuscript in preparation). So far, however, we have not been able to serologically detect the expression of these on the clones by an antibody against a T cell receptor gene product (K. Okumura, personal communication). At present, there is no definitive evidence whatsoever that NK cells functionally use T cell receptors, even though C_β genes are rearranged in most cases (47). Indeed, in our preliminary results, all the LGL clones showed the same pattern of C_β gene rearrangement, with no apparent correlations to functional heterogeneity of their cytotoxic repertoires. Although these data support the idea that LGL-NK may belong to T cell lineage, we feel that the question of the NK recognition system remains essentially unresolved.

Although the exact significance of BMC-directed cytotoxicity by NK cells has remained obscure, several recent reports (22, 23) indicate that a normal NK-enriched population could functionally affect various stages of normal lympho-hematopoietic progenitor cells. Present data indicate that cloned LGL can indeed suppress the development of hematopoietic colonies (CFU-C) from normal BM. In normal situations, however, such autoreactivity by LGL may be potentially harmful to the host, unless it is well regulated. Our present results reveal that normal macrophages suppress the intrinsic autoreactivity of LGL clones against normal BMC. Thus, LGL clones maintained with macrophages showed much less cytotoxic activity for BMC, as well as less inhibitory activity for in vitro hematopoiesis than those without. On the contrary, the clones similarly cultured with macrophages showed rather potentiated cytotoxicity against malignant or virus-infected targets (Table III). Although not presented here, our preliminary results show that high concentrations of IL-1 can also replace this functional effect of macrophages. The mechanism of differential regulation of the activity of a single clone by macrophages is not clear at present. We have evidence suggesting that distinct arachidonate metabolites are in some way critically involved in the expression of cytotoxicity of LGL clones, cyclooxygenase pathway for BMC targets, and lipoxygenase pathways for leukemias and virus-infected cells. It thus seems possible that metabolic priming of LGL by macrophages in terms of arachidonate metabolism is somehow involved in the functional shift. In any case, the observation suggests that macrophages might endogenously regulate NK activity in such a way that potential autoreactivity of NK cells is consistently suppressed.

In conclusion, present results seem to represent the physiological regulation of both growth and function of endogenous NK cells by macrophages in vivo. Such regulation would provide the NK effector system an immediate responsiveness to IL-2 without prior stimulation, as well as a certain functional selectivity by which abnormal cells are affected and normal vulnerable cells protected from the intrinsic autoreactivity of NK cells.

Summary

Using cloned lines with the morphology of large granular lymphocytes (LGL) from BALB/c mice, we studied the exact requirements for proliferation and their functional characteristics, as well as their regulation. Although these cloned LGL lines were interleukin 2 (IL-2) dependent for growth, experiments using human recombinant IL-2 (rIL-2), known to be active on murine cells, indicated

that IL-2 was a necessary but not sufficient factor. Coexistance of normal macrophages in addition to rIL-2 was found to support continuous proliferation of cloned LGL in vitro. This role of macrophages could be replaced by partially purified IL-1 derived from macrophage-conditioned medium. An IL-2 binding assay using ^{125}I-rIL-2 suggested that the role of normal macrophages was to selectively induce and/or maintain high affinity IL-2 receptors (IL-2R) (K_d, 0.2–0.5 nM) without affecting low affinity ones (K_d, 10–30 nM).

Functional studies indicated that most of the LGL clones killed various combinations of representative groups of natural killer (NK)-susceptible target cells, including leukemic cells (YAC-1, RLδ1), virus-infected cells (HeLa-measles, HeLa–herpes simplex virus), and normal bone marrow cells (BMC), whereas none of them affected any of NK-resistant target cells, including uninfected HeLa cells. Some of these clones also suppressed in vitro hematopoiesis. Such characteristic cytotoxic spectra, as well as serological phenotypes (Thy-1$^+$, Lyt-1$^-$2$^-$, asialo GM$_1$–positive, T200$^+$, TdT$^-$, Fc receptor–positive) indicated that these LGL clones exactly represent endogenous NK cells, rather than a variety of anomalous killer cells generated in various culture conditions. Although there was significant heterogeneity of cytotoxic spectrum among LGL clones, no clonotypic distribution of specificities was observed. Normal macrophages were found to modulate the functional expression of LGL clones. They augmented the cytotoxic potential of the clones against leukemic and virus-infected targets, but suppressed intrinsic reactivity against normal BMC. Similarly, LGL clones maintained with macrophages showed much less suppressive effect on in vitro hematopoiesis. The present observations on the interaction of cloned LGL and normal macrophages provide a basic explanation for the mechanisms by which the immediate responsiveness to IL-2 of the NK effector system, without exogenous stimulation, and the functional selectivity toward abnormal rather than normal cells, are actively maintained in vivo.

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