The preleukemic state of mice reconstituted with Mixl1-transduced marrow cells

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Murine granulocytic cells, in becoming leukemic, need to acquire enhanced self-generation and a capacity for autocrine growth stimulation. Mice transplanted with bone marrow cells transduced with the Mixl1 homeobox gene develop a very high frequency of myeloid leukemia derived from the transduced cells. Preleukemic mice contained a high frequency of transduced clonogenic granulocytic cells. They exhibited an abnormally high capacity for self-replication and could generate immortalized granulocytic cell lines that remained absolutely dependent on either GM-CSF or IL-3 and were not leukemic. Organs from mice repopulated by marrow cells transduced either with Mixl1 or the control murine stem cell virus vector exhibited a capacity to produce IL-3 *in vitro*, activity being highest with the lungs, marrow, bladder, and thymus. Supporting evidence for the *in vivo* production of IL-3 was the frequent development of mast cells in the marrow. Overexpression of Mixl1 appears capable of inducing an abnormal self-renewal capacity in granulocytic precursors. Aberrant production of IL-3 was not present in the continuous Mixl cell lines and was therefore not in itself likely to be a leukemogenic change but it could support the enhanced survival and proliferation of the Mixl1 granulocytic populations until a final leukemogenic mutation occurs in them.

IL-3 | myeloid preleukemia | self-renewal

Studies on murine models of myeloid leukemia development have indicated that two major changes need to occur in the granulopoietic cells undergoing transformation. These changes are the development of an abnormally elevated level of self-generation and the development by one means or another of autocrine growth stimulation (1, 2).

Overexpression of various clustered homeobox genes such as *Hoxa9*, *HOXA10*, *HOXB3*, *HOXB6*, or *HOXB8* has been shown to be a potent induction system for myeloid leukemia development (3). More recently, overexpression of nonclustered homeobox genes, *Cdx2* and *Mixl1*, has also been shown to induce myeloid leukemia development in mice (4, 5).

Overexpression of Hox8 (Hox2.4) was found to conditionally immortalize myelomonocytic, megakaryocytic, and mast cell progenitors (6). Similarly, overexpression of HOXB6 expanded the numbers of hematopoietic stem cells and myeloid precursors, which could then be sequentially recloned *in vitro* as clonal populations of blast cells. This process was subsequently followed by the development of myeloid leukemia (7). However, overexpression of HOXB4 merely caused expansion of the hematopoietic stem cell population and enhanced proliferation of myeloid cells without leading to leukemia development (8).

The very high frequency of myeloid leukemia developing in mice repopulated by marrow cells overexpressing Mixl1 (5) offered an ideal opportunity for examining the granulopoietic population during the preleukemic period to determine whether either of the above changes linked to transformation had been induced by Mixl1 overexpression.

The results indicated that Mixl1 overexpression induces an expansion of somewhat atypical granulopoietic cells in the preleukemic period. These exhibit an abnormal capacity for self-generation and the formation of continuous lines of growth factor-dependent granulocytic cells that were not leukemic. In parallel, the organs from mice repopulated by Mixl1- or murine stem cell virus (MSCV)-transduced marrow developed a capacity to produce IL-3. This was not a leukemogenic event but the aberrant IL-3 production could be proposed to allow the survival and expansion of Mixl1-altered cells permitting them to acquire one or more subsequent leukemogenic mutations.

Results

In response to stimulation by GM-CSF, GFP+ marrow or spleen cells from preleukemic Mixl1 mice aged 2–9 months generated an elevated number of granulocytic colonies that were abnormal in shape and composition. These populations of GFP+ cells also produced blast cell colonies, not a colony type normally stimulated by GM-CSF (Table 1) (9). A similar pattern was evident in cultures stimulated by IL-3. The GFP positivity of the cells cultured certified transduction by the Mixl1 construct. In contrast, the culture of GFP+ cells from mice repopulated by cells transduced with control MSCV virus showed a normal pattern of colony formation in response to GM-CSF (Table 1) and IL-3.

In preliminary control recloning experiments, normal C57BL marrow cells were stimulated by GM-CSF and, after 7 days of incubation, granulocytic, granulocyte–macrophage, and macrophage colonies were resuspended and recultured in medium, again with stimulation by GM-CSF. As shown in Table 2, with the media in use, none of the colonies contained cells that produced granulocytic or granulocyte–macrophage daughter colonies in secondary culture. Many colonies produced low numbers of small macrophage colonies but they, on reculture, were unable to produce further daughter colonies.

The production of even small numbers of macrophage colonies by cells from what appeared to be pure granulocytic colonies was surprising. To explore this finding further, colony-sized intercolony areas of the agar cultures adjacent to colonies were harvested, resuspended, and recultured. These intercolony areas produced similar numbers of small macrophage colonies to those produced by recloned granulocytic colonies (Table 2), indicating that the unexpected results with recultured granulocytic colonies were likely to have been caused by contaminating clonogenic cells in intercolony areas that had become incorporated by chance, in some expanding granulocytic colonies.

The results of reculturing GM-CSF-stimulated colonies grown from GFP+ fractions of marrow cells from mice repopulated by Mixl1-transduced marrow cells were strikingly different. Based on the type of data shown in Table 1, the colonies selected for reculture were compact blast colonies or granulocytic colonies of unusual shape or large size. Because of the difficulty in unambiguously distinguishing some blast from granulocytic colonies, results using both types of colony have been pooled. Of the 65 such colonies recultured, 14 were likely to have been blast colonies and 51 possibly granulocytic colonies. As shown in

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Table 1. Primary cultures used as donors in colony recloning experiments

Cells cultured	No. of marrows cultured	No. of colonies				
		Blast	Granulocytic	Granulocytic-macrophage	Macrophage	Eosinophil
GFP + Mixl1 preleukemic	5	32 ± 29	91 ± 22	12 ± 9	10 ± 13	0 ± 0
Control GFP + MSCV transduced	4	0 ± 0	18 ± 13	3 ± 3	18 ± 9	1 ± 1
C57BL bone marrow	4	0 ± 0	10 ± 4	5 ± 2	12 ± 4	2 ± 1

All cultures contained 2,000–10,000 cells, were stimulated by 10 ng/ml GM-CSF, and analyzed after 7 days of incubation. For recloning studies cultures of Mixl1 GFP+ cells contained only 2,000 cells per culture, whereas control cultures contained 10,000 cell per culture. For comparison, colony numbers have been standardized to numbers per 10,000 cells. Mean colony numbers ± SDs.

Table 2, the behavior of these colony cells on reculture was the exact opposite of that of normal colonies. M-CSF-stimulated no colony formation and, in particular, no macrophage colony formation. In the secondary cultures, GM-CSF also failed to stimulate the formation of any macrophage-containing colonies. What GM-CSF did stimulate was the formation of blast colonies from 11% of the colonies (three from colonies with blast shape and four from colonies that were possibly granulocytic) and granulocytic colonies from 72% of the colonies. The numbers and size of secondary colonies varied widely but secondary blast colonies retained the compact or multicentric appearance of blast colonies in primary cultures.

Generation of Continuous Cell Lines. When such secondary Mixl1 colonies were resuspended and recultured, further daughter colonies were produced, and this sequence was able to be repeated for at least six sequential reculture cycles (data not shown). The results clearly documented an abnormal capacity of Mixl1 blast and granulocytic colony-forming cells to self-generate. Mixl1 GFP+ colonies were therefore tested for their capacity to generate continuous cell lines, using colonies initiated either by GM-CSF or IL-3. In each case the picked-off colony was thereafter stimulated by the agent used to initiate colony formation.

In an initial series of control cultures, 30 granulocytic or granulocyte–macrophage colonies initiated by GM-CSF in cultures of normal C57BL marrow cells were placed in individual culture wells. After 2 months of stimulation by GM-CSF, only five of these wells possibly contained actively dividing cells, whereas the remainder either contained static cell populations or dying cells. Cytocentrifuge analysis of 29 of these cultures at 2 months showed that none contained granulocytic or blast cells, the only cells present being unhealthy macrophages. On this basis the cultures were terminated.

Colonies stimulated to develop by either GM-CSF or IL-3 were grown from GFP+ Mixl1 transduced marrow cells, and the largest were tested for their capacity to generate continuous cell lines. Of these, 25% of GM-CSF-stimulated colonies and 33% of IL-3-stimulated colonies were able to generate continuously growing clonal cell lines that have now been in continuous GM-CSF- or IL-3-stimulated culture for >12 months. Each cell line contains some blast cells together with a variable proportion of granulocytic cells at various stages of differentiation. Fig. 1*A* shows three such cell lines with varying levels of differentiation. All cells in the cell lines were GFP+, and a significant fraction of the cells were Mac-1+, but the cells were negative for GR1, a feature of Mixl1-induced myeloid leukemic populations (5). Southern analyses of the cell lines indicated that each contained one to three copies of the Mixl1-transduced gene.

It was of interest that an additional 14 continuous cell lines (10 maintained with IL-3, 4 with GM-CSF) were not derived from colonies grown from preleukemic mice but from post-5-fluorouracil (5FU) marrow cells transduced *in vitro* with Mixl1 then merely cultured for 2 days with growth factor stimulation. These cells were not fractionated to obtain GFP+ cells but the unfractionated cells were used to generate colonies in agar. Colonies that were large and somewhat atypical in appearance were found to be able to generate continuous cell lines that subsequent analysis showed were GFP+. Southern analysis of retroviral integration in these latter cell lines indicated that three lines from the same liquid culture were related and likely to have been generated from one single initiating cell within the 2-day liquid incubation period. These data suggest that Mixl1 transduction must be able to confer immortalization almost immediately.

The frequency of clonogenic colony-forming cells in the cell lines could be quite high, and cell lines maintained on GM-CSF formed more colonies when stimulated by GM-CSF than when stimulated by IL-3. The reverse was true for cell lines maintained with IL-3 (Fig. 1*B*). Some colonies were composed of blast cells, but others were composed of maturing, if somewhat abnormal, granulocytes. In addition, some clonogenic cells in the lines were capable only of generating small clusters. The behavior of the cell lines in clonal culture suggested that the lines were maintained by blast cells with continuous loss of cells from the population by maturation in the granulocytic lineage to cells with lower proliferative potential and eventually to nondividing cells.

None of the cell lines responded by proliferation in clonal cultures when stimulated by G-CSF, M-CSF, stem cell factor (SCF), Flk ligand (FL), or IL-6 and the addition of leukemia inhibitory factor (LIF), IL-6, or oncostatin M (OSM) to cultures stimulated, either by GM-CSF or IL-3, did not alter colony numbers or maturation.

No clonogenic cells proliferated in unstimulated cultures, and when 10^6 cell line cells were injected s.c. or i.v. to groups of four normal C57BL recipients, no transplanted leukemias developed in the following 6 months.

Production of IL-3. When normal mouse organs are minced then cultured in serum-free or serum-containing medium, no IL-3 production has been observed with BaF3 cells as the detection system, nor has IL-3 mRNA been detected by PCR analysis (10).

It was therefore very surprising that marrow cells from most preleukemic mice repopulated by marrow cells transduced with

Table 2. Progenitor cell content of GM-CSF-stimulated colonies

Primary cultures	Colony type recloned	Stimulus in secondary culture (no. of colonies tested)	No. of negative colonies	Progenitor cells in positive colonies							
				Blast		Granulocytic		Granulocyte-macrophage		Macrophage	
				No. positive	Mean progenitor nos. per positive colony	No. positive	Mean progenitor nos. per positive colony	No. positive	Mean progenitor nos. per positive colony	No. positive	Mean progenitor nos. per positive colony
Normal C57BL marrow	Granulocytic	GM-CSF (49)	45	0	—	0	—	0	—	4	20 ± 19
		M-CSF (38)	33	0	—	0	—	0	—	5	48 ± 41
	Granulocyte-macrophage	GM-CSF (29)	13	0	—	0	—	0	—	16	26 ± 24
		M-CSF (21)	7	0	—	0	—	0	—	14	64 ± 66
	Macrophage	GM-CSF (46)	14	0	—	0	—	0	—	32	21 ± 18
		M-CSF (33)	2	0	—	0	—	0	—	31	57 ± 48
	Colony-free agar	GM-CSF (23)	20	0	—	0	—	0	—	3	8 ± 0
		M-CSF (23)	16	0	—	0	—	0	—	7	15 ± 7
GFP + Mix11 marrow	Blast or granulocytic	GM-CSF (65)	32	7	3 ± 17	47	24 ± 43	0	—	0	—
		M-CSF (30)	30	0	—	0	—	0	—	0	—
GFP + MSCV marrow	Granulocytic or granulocyte-macrophage	GM-CSF (10)	10	0	—	0	—	0	—	0	—

All primary cultures were stimulated by 10 ng/ml GM-CSF and individual colonies were removed after 7 days of incubation. Mean colony numbers ± SDs.

Fig. 1. Mix1-transduced marrow cells form continous cell lines. (*A*) Three cloned GFP+ Mixl1-transduced continuous granulocytic cell lines with varying degrees of differentiation are shown. The stain used was May-Grümwald Giemsa. (Magnification: ×500.) (*B*) Number of colonies formed after 7 days in agar culture by 200 cells from seven cell lines maintained with GM-CSF and seven cell lines maintained with IL-3 is shown. Note that lines maintained with GM-CSF responded better to GM-CSF (GM) than to IL-3 and vice versa.

Mixl1 produced detectable IL-3 (Table 3) as did most marrows from mice repopulated by control MSCV-transduced marrow cells (Table 3). This finding was not paralleled by any unusual capacity to produce GM-CSF, the other regulator able to stimulate Mixl1-transformed cells. Indeed, the Mixl1- and MSCV-transformed marrows had a subnormal capacity to produce even the low levels of GM-CSF normally produced by control marrow cells.

These assays were extended to examine possible IL-3 production by other organs from these repopulated mice. As shown by the typical example in Fig. 2*A*, IL-3 production was observed with a number of organs from both types of mouse, the most consistent and active being the lung, bladder, marrow, thymus, and uterus. Oddly, the marrow shaft, which is usually far more active than the marrow in the production of other cytokines (10, 11), was usually relatively or completely inactive in IL-3 production.

The specificity of the BaF3 assays on the conditioned media was confirmed by showing that mAb to IL-3 completely blocked the stimulating activity of these conditioned media (Fig. 2*B*).

To further explore the origin of this bizarre production of IL-3, mice were examined after various steps of the transduction–repopulation procedure. As shown in Table 3, with one dubious exception, organs from mice injected with 5FU did not produce IL-3. In the case of irradiated mice transplanted with normal C57BL marrow cells low levels of IL-3 production were observed in only 2 of 47 mice. This frequency was unchanged in irradiated mice injected with post-5FU marrow cells with or without initial culture *in vitro* (Table 3) (see also examples in Fig. 2*A*).

These results indicated that irradiation and repopulation resulted infrequently in a weak capacity of some organs to

Table 3. Effect of repopulation by marrow cells transduced with Mixl1 or MSCV on capacity of the marrow to produce IL-3 and contain mast cells

Procedure	Repopulated by	No. of positive marrows / no. tested		
		GM-CSF production	IL-3 production	Mast cell content
Irradiation	Mixl1-transduced	2/23	17/23	7/13
Irradiation	MSCV-transduced	2/22	20/22	9/15
Irradiation	5FU-treated	1/4	0/4	0/4
Irradiation	Normal marrow	16/47	2/47	0/34
5FU	—	16/20	0/20	—
None	—	10/29	0/29	0/16

Mice were examined 2–12 months after repopulation or 1–7 days after 5FU treatment.

produce IL-3. However, the uniform striking production of IL-3 by various organs of recipient mice appeared to be the result of the transduction procedure on the donor marrow, whether using the Mixl1 or control MSCV viruses.

In sharp contrast, IL-3 production was not detected in repeat assays on media from cultures of any of 21 continuous granulocytic cell lines derived from Mixl1 marrow cells. If these cells are representative of the dominant granulocytic population developing in mice receiving Mixl1-transduced cells, then such cells seem not to have been responsible for the IL-3 production observed with preleukemic Mixl1 marrow. Furthermore, MSCV-transduced mice do not contain altered populations in the marrow, yet these marrows were equally active in producing IL-3. Hematopoietic cells also would seem to be an unlikely cellular source of IL-3 production by organs such as the lung or bladder, the more so because no organs other than the marrow or spleen contained collections of hematopoietic cells in the preleukemic Mixl1 mice.

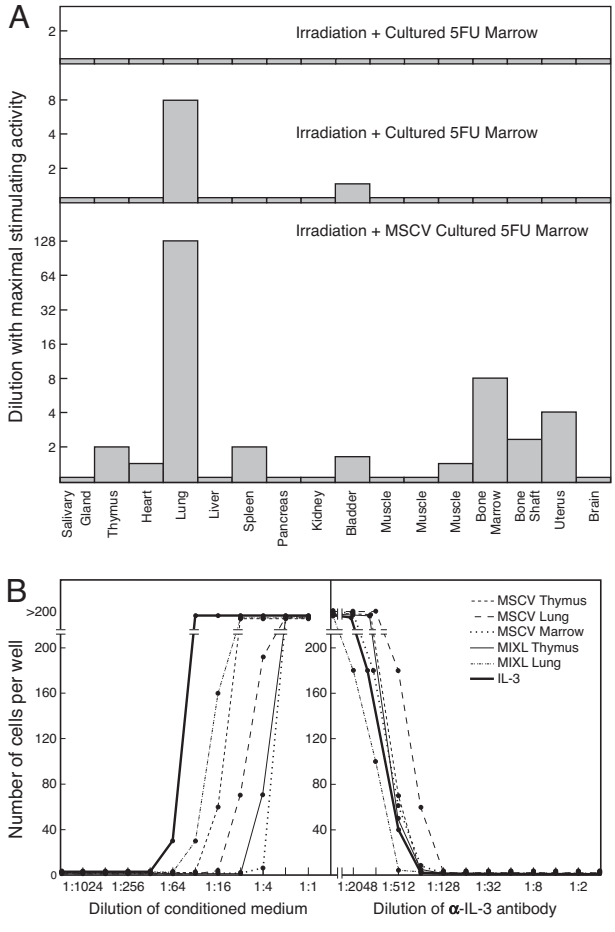

Fig. 2. Organs from transplanted mice produce IL-3. (*A*) Shown are results of microwell assays using BaF3 cells of IL-3 concentrations in medium conditioned by various organs from two irradiated mice injected with cultured post-5FU marrow cells and one irradiated mouse injected with MSCV-transduced post-5FU marrow cells. Columns indicate the highest dilution of medium causing maximal proliferation of the BaF3 cells. Organs were analyzed 2 months after transplantation. (*B*) (*Left*) Typical microwell titration assays of organ-conditioned medium on BaF3 cells. (*Right*) All stimulating activity was neutralized by a mAb against IL-3.

The production *in vitro* of cytokines by cultured organs involves a strong *in vitro* initial induction of transcription by as-yet-unidentified mechanisms (10), and the demonstration of IL-3 production *in vitro* does not necessary certify that these organs were producing IL-3 *in vivo*. Mast cells are the most responsive population to the *in vivo* injection of IL-3. The population of mast cells was increased 100-fold in the spleen after a short course of IL-3 injections, but mast cells did not develop in the marrow of such mice (12). However, when irradiated mice were repopulated by marrow cells transduced with, and overexpressing, IL-3 cDNA, large numbers of mast cells developed in the marrow (13).

On this basis, sections of sternal marrow from mice repopulated by the various types of marrow cells were stained with toluidine blue. As shown in Table 3, in normal C57BL control mice no sternal marrow contained mast cells. Similarly, in sections of the sternum from 34 irradiated mice repopulated by C57BL marrow cells again none contained mast cells. In contrast, in the sternum from 15 irradiated mice repopulated by MSCV-transduced marrow, 9 (60%) contained mast cells (Fig. 3) (Table 3). Similarly, in sternal sections from 13 mice repopulated by Mixl1-transduced marrow cells, 7 (54%) contained varying numbers of mast cells. The presence of mast cells in these marrows represents strong evidence that the marrow populations in these mice were indeed producing IL-3 *in vivo*.

Discussion

Previous studies have documented two essential steps in the development of myeloid leukemia in mice: (*i*) the development of an abnormally high capacity for self-generation in immature cells of the cell population undergoing leukemogenesis, and (*ii*) the acquisition by one mechanism or another of a capacity for autocrine proliferative stimulation (1, 2). The model system in which marrow cells are transduced with and overexpress the Mixl1 homeobox gene is highly efficient in leading to the development of myeloid leukemia, and any mice examined before leukemia develops can be accepted as being in a preleukemic state (5).

In the present studies, mice repopulated by Mixl1-transduced marrow cells were analyzed in the preleukemic period to deter-

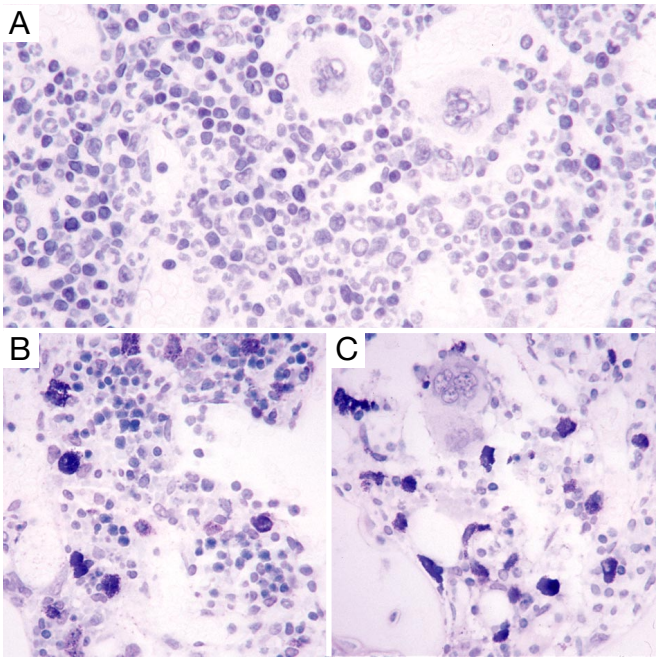

Fig. 3. Mast cells develop in the marrow of mice transplanted with transduced marrow cells. (*A*) Normal mouse bone marrow stained with toluidine blue and showing no metachromatic mast cells. (*B* and *C*) Two marrows repopulated by marrow cells transduced with MSCV (*B*) or Mixl1 (*C*) showing numerous metachromatic mast cells. (Magnification: ×200.)

mine whether either or both of the necessary changes leading to leukemia development had developed after repopulation.

All methods of analysis of preleukemic mice (cytocentrifuge, FACS analysis, or histological sections) documented a progressive rise in granulocytic populations usually occurring first in the marrow and then in the spleen at the expense of preexisting erythroid and lymphoid populations (5). Granulocytic populations were never found in the liver, distinguishing the preleukemic state from that characteristic of myeloid leukemia in mice. Culture of marrow and spleen cells from the preleukemic mice documented a many fold rise in granulocytic progenitors that formed somewhat abnormal, but maturing, granulocytic progeny. Also present were blast cell colonies that were also abnormal in being responsive to stimulation by GM-CSF, in addition to IL-3 - the usual stimulus for blast colony formation.

Recloning of Mixl1 blast and granulocytic colonies showed them to have an abnormally high content of colony-forming cells and further that such colony-forming cells could be repassaged for at least six sequential reculture cycles. This finding contrasted with the absolute inability of normal granulocytic colony-forming cells to generate any granulocyte colony-forming progeny. As suggested by these results, it proved relatively easy to derive cloned cell lines from 25–30% of these blast and granulocytic colonies, and these have remained in continuous culture either with GM-CSF or IL-3 for >12 months. These candidate immortalized cell lines exhibit continuous maturation to relatively mature neutrophils; the cells remain GFP+ but are GR1−. The cell lines remain absolutely dependent on GM-CSF or IL-3 for survival and respond by proliferation to no other cytokine. Furthermore, they are not affected by agents such as IL-6, LIF, or OSM that are able to induce maturation and/or suppress proliferation in some leukemic cell lines (14, 15). The cell lines have remained nonleukemogenic on transplantation to normal syngeneic recipients.

These experiments have documented the development in preleukemic Mixl1 mice of an abnormal blast/granulocytic population capable of extensive self-generation and with some cells possibly having been immortalized. Thus, these preleukemic mice have clearly acquired one of the two changes necessary for leukemogenesis. It is not known whether multiple clones of such self-generating cells are present in individual mice, but several continuous cell lines derived from the same preleukemic animal appear to have differing insertion patterns for the Mixl1 cDNA, implying the presence of multiple self-generating clones in individual preleukemic mice.

The demonstration that preleukemic Mixl1 mice were producing IL-3 was quite unexpected, but no more so than the finding that control-transduced mice were also producing IL-3. The mechanism involved had some relation to viral transduction then repopulation, but why the IL-3 gene becomes activated remains unclear.

What is important is that the IL-3 production seems not to occur in the affected blast/granulocytic population as judged from the negative results with the Mixl1 continuous cell lines. The abnormality is not therefore a transforming event because such an event requires autocrine stimulation, a conclusion supported by the observed IL-3 production by control MSCV-transduced mice that do not develop abnormal granulocytic populations or myeloid leukemia.

What the IL-3 production may achieve is the maintenance and expansion of the abnormally self-reproducing blast/granulocytic populations, allowing a second mutational event to occur in one or more cells in the preleukemic population. This situation would be similar to that documented in earlier studies. Immortalized, but nonleukemic, FDCP1 cells depend for survival and proliferation on either GM-CSF or IL-3 (10). When such cells were engrafted into preirradiated mice or transgenic mice with excess GM-CSF levels, the FDCP1 cells presumably were allowed to persist and increase in numbers by the irradiated milieu or the excess GM-CSF levels. However, when leukemic transformation finally did occur in these cells it always involved acquisition of an autocrine capacity to produce either GM-CSF or IL-3 caused by activating rearrangements of either gene (16, 17). It is unclear why autocrine growth factor production should differ so radically from extrinsic growth factor stimulation, but possibly if the GM-CSF or IL-3 should meet endosomally displayed receptors in the cytoplasm sustained or aberrant signaling might result and constitute an autocrine proliferative change able to serve as a second event in leukemogenesis.

Materials and Methods

Generation of Retroviruses. An MSCV-based retrovirus containing the internal ribosome reentry site from the encephalomyocarditis virus and GFP cDNA (18) was modified by insertion of the sequence encoding a FLAG epitope and a Mixl1 restriction site into the multiple cloning site. Mixl1 cDNA was cloned into the Mlu1 site. Retroviral supernatants were collected from Phoenix E cells (19) at 48 and 72 h after transfection, and media were filtered and stored at −80°C until use.

Marrow Transduction and Transplantation. Marrow cells were harvested from C57BL6 donor mice 4 days after the i.v. injection of 150 mg per kg of 5FU and cultured for 24 h in DMEM with 10% FCS, 100 ng/ml murine SCF, 6 ng/ml IL-3, 10 ng/ml IL-6, 4 ng/ml IL-1B, and 1 ng/ml IFN-γ. Then 1–2 × 10^6 cells/ml in the same media were transferred to 12-well plates coated with Retronectin (Takara Bio), and retroviral supernatant (50% vol/vol) and polybrene (6 μg/ml) were added. After two rounds of spinfection lasting 2 days (20), 0.25–1 × 10^6 cells were injected i.v. to irradiated (2 × 5.5 Gy) C57BL mice. Reconstitution was monitored 12 weeks after transplantation by determining the percentage GFP+ cells in retroorbital blood.

Culture. Routinely, 25,000 marrow or 50,000 spleen cells were cultured in 35-mm Petri dish cultures containing 1 ml of DMEM with 20% FCS and 0.3% agar (21). In replicate cultures, the following final concentrations of stimuli were used: murine GM-CSF (10 ng/ml), human G-CSF (10 ng/ml), human M-CSF (10 ng/ml), murine IL-3 (10 ng/ml) or murine SCF (500 ng/ml) + IL-3 (10 ng/ml) + erythropoietin (Epo) (2 units/ml). After 7 days of incubation at 37°C in a fully humidified atmosphere of 10% $C0_2$ in air, colonies were scored by using an Olympus dissection microscope. Then the cultures were fixed for 4 h with 1 ml of 2.5% glutaraldehyde, floated intact onto glass slides, allowed to dry, and then stained in sequence for acetylcholinesterase, Luxol Fast Blue, and hematoxylin (21). All colonies in these cultures were then typed at ×100 or ×200 magnifications.

Recloning or Continuous Cell Lines. For recloning studies, cultures containing only 2,000 cells per/ml were used to ensure clear separation of the colonies being recultured. Individual 7-day colonies from cultures stimulated by 10 ng/ml GM-CSF were removed with a fine pipette, resuspended by pipetting in 6–8 ml of agar medium, then recultured in duplicate cultures using GM-CSF, IL-3 or SCF + IL-3 + Epo. After an additional 7 days of incubation, colonies were scored and the reculture procedure was repeated.

To generate continuous cell lines, the largest colonies developing in GFP+ Mixl1 marrow cell cultures stimulated either by 10 ng/ml GM-CSF or 10 ng/ml IL-3 were individually placed in 1-ml well cultures containing DMEM with 10% FCS and either 10 ng/ml GM-CSF or 10 ng/ml IL-3. As necessary, cultures were split and recultured with fresh medium and GM-CSF or IL-3 once or twice per week. In additional experiments, the colonies used to generate continuous cell lines were grown from Mixl1-transduced marrow cells that had not been subjected to GFP fractionation before culture in agar.

Preparation and Assay of Organ-Conditioned Medium. Organs were removed sterilely from irradiated mice repopulated 2–12 months previously by Mixl1- or MSCV-transduced marrow cells or by normal C57BL marrow cells. In additional experiments, mice used had been injected with 150 mg/kg 5FU 1–7 days previously.

Each organ was minced with scissors then incubated for 4 days in 2 ml of DMEM containing 10% FCS. Conditioned media were harvested from these cultures, filtered, then assayed in duplicate serial 2-fold dilutions in microwell cultures of 200 BaF3 cells (10). After 48 h of incubation, the numbers of viable cells in the microwell cultures were scored by using an inverted microscope.

In control studies, 5 μl of appropriate concentrations of conditioned media was mixed with 5 μl of titrations of monoclonal IL-3 antibody (PeproTech) to determine whether the antibody completely inhibited all stimulating activity for BaF3 cells.

Flow Cytometry. Analysis and sorting were performed by using a Di Va high-speed sorter or an LSR flow cytometer (BD Biosciences) with reagents as described (5).

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