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Correlation of cell-surface phenotype with the establishment of interleukin 3-dependent cell lines from wild-mouse murine leukemia virus-induced neoplasms

(retrovirus)

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ABSTRACT **The wild mouse ecotropic virus, Cas-Br-M murine leukemia virus, induces myeloid and erythroid leukemias as well as T-cell and B-cell lymphomas in NFS mice. The ability to establish long-term cell lines from these tumors in the presence or absence of the T-cell-derived lymphokine interleukin 3 (IL-3) was examined. IL-3-dependent cell lines were readily obtained from the majority of the myeloid or erythroid leukemias and immunoblastic lymphomas. In the absence of IL-3, only one long-term factor-independent cell line was obtained from a myelogenous leukemia. The majority of the thymic T-cell lymphomas or B-lineage lymphomas could not be cultured in the presence or absence of IL-3. The results suggest that transformation of hematopoietic lineages does not necessarily obviate the requirement for normal growth factors. The acquisition of independence from growth factors may require additional transforming events.**

A variety of factors contribute to the induction of leukemia by murine retroviruses. One requirement is a cellular immune response (1–3). The immune response has been postulated for the generation of target cell populations for transformation. In viremic mice, viral antigen-specific helper T cells produce lymphokines such as interleukin 3 (IL-3), interleukin 2 (IL-2), and granulocyte/macrophage colony-stimulating factor, resulting in a 50- to 100-fold increase in the frequency of cells that are under the regulation of these growth factors (4).

IL-3 was identified by its ability to induce the T-cell-associated enzyme 20α-hydroxysteriod dehydrogenase in cultures of *nu/nu* splenic lymphocytes (5–7). By using this assay, IL-3 was purified to homogeneity and was shown to be a glycoprotein of 28 kDa (8, 9). More recently, cDNA clones have been obtained that encode IL-3 (10, 11). Purified IL-3 mediates a variety of biological effects *in vitro*, including induction of mast cell differentiation, burst-promoting activity, colony-stimulating activity, stem cell activating activity, and induction of Thy-1 expression (9, 12). The spectrum of biological activities is due to the ability of IL-3 to induce the proliferation of an early hematopoietic/lymphoid stem cell.

A relationship of primary retrovirus-induced tumors to IL-3-regulated differentiation was suggested by the observation that Moloney murine leukemia virus (MoMuLV)-induced lymphomas express 20α-hydroxysteroid dehydrogenase (6) and often require IL-3 for growth *in vitro* (1). To determine whether establishment of IL-3-dependent cell lines is correlated with primary tumor phenotype, 57 primary Cas-Br-M MuLV-induced tumors were assessed for their ability to grow *in vitro* in the presence or absence of IL-3. Cas-Br-M is a wild-mouse-derived MuLV that induces a wide spectrum of hematopoietic neoplasms (13). The results demonstrate that IL-3-dependent cell lines were most often established from myeloid and erythroid leukemias and from immunoblastic lymphomas.

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MATERIALS AND METHODS

Cas-Br-M MuLV-Induced Neoplasms. Cell-free extracts of neoplastic spleens or lymph nodes from mice inoculated with Cas-Br-M ecotropic virus were injected intraperitoneally into newborn NFS/N mice. Also included in this study were neoplasms (NFS-58, -60, and -61) of (NFS × DBA/2) F_1 or (NFS × NFS.C58 v-1) F_1 mice that had been inoculated with Cas-Br-M ecotropic virus or a wild mouse ecotropic virus, C2S, or MCF isolates of C2S-infected mice (J. Hartley, personal communication).

Cell Culture. Single cell suspensions were made from neoplastic spleens and were resuspended at 2×10^6 cells per ml in RPMI 1640 medium and 10% fetal calf serum containing penicillin and streptomycin or in medium containing 20 units of purified IL-3 per ml or 25% WEHI-3 conditioned medium (CM) as a source of IL-3. The WEHI-3 CM was prepared as described (15).

Flow Cytometry. Cell lines were stained as described (16); nonviable cells were removed by centrifugation through a Ficoll/Hypaque gradient. Antibodies to Thy-1.2, Ly-5(B220), ThB, Ly-5, "8C5," and a goat anti-mouse immunoglobulin were used labeled directly with fluorescein isothiocyanate (FITC). Antibodies to Ly-1.2, Ia^k, Ly-17, and Lyb-2.1 were counterstained with a FITC-labeled anti-mouse IgG_2; Mac-1, Ly-24, and "H-11" were counterstained with FITC-labeled rabbit anti-rat immunoglobulin. Specificity of staining was determined by comparison with counterstain-only controls. Normal spleen cells were used as controls. Cells were analyzed on a fluorescence-activated cell sorter (FACS II, Becton Dickinson). Nonviable cells were electronically gated from analysis by light scatter and uptake of propidium iodide (17).

Radioreceptor Assay. The procedures used for the detection of receptors for IL-3 have been described in detail (18).

Other Techniques. IL-3 was purified to homogeneity and iodinated as described (8, 9).

RESULTS

Establishment of IL-3-Dependent and -Independent Cell Lines. The spectrum of hematopoietic tumors induced in NFS/N mice by Cas-Br-M MuLV is illustrated in Table 1. Among 57 primary neoplasms, there were 29% thymic

Abbreviations: IL-2 and -3, interleukins 2 and 3; MoMuLV, Moloney murine leukemia virus.

Table 1. Establishment of cell lines from Cas-Br-M MuLV-induced tumors

	No.	No growth	IL-3 limited growth (3–5 months)	IL-3-dependent lines	Factor-independent lines	Factor-independent limited growth (3–5 months)
Thymic lymphoma	17	8	5	1	1	2
Myelogenous leukemia	12	1	2	8	1	0
Mixed follicular center cell lymphoma	2	0	1	1	0	0
Erythroleukemia	6	1	1	3	0	1
Immunoblastic lymphoma	4	0	1	3	0	0
Lymphoblastic lymphoma	16	7	4	3	2	0
Total	57	17	14	19	4	3

Primary Cas-Br-M MuLV-induced tumors were classified by standard histopathological criteria (13). Growth was characterized as either limited, in which there was a substantial growth for 3–5 months but a cell line was not established, or as a growth that gave rise to a cell line.

lymphomas, 28% B-cell lymphoblastic lymphomas, 7% B- or T-cell immunoblastic lymphomas, 3% follicular center cell lymphomas, 21% myelogenous leukemias, and 11% erythroleukemias. In each case, single cell suspensions were cultured in either medium or medium supplemented with IL-3 and evaluated for growth (Table 1). Among the 57 primary neoplasms, 17 (30%) showed no growth *in vitro* in either the presence or absence of IL-3. The remainder of the cultures showed either limited growth for 3–5 months or gave rise to long-term cell lines. Limited growth was observed with 14 (25%) of the tumors in cultures supplemented with IL-3 and an additional 3 tumors showed limited growth in the presence or absence of IL-3. In some cases, the limited growth in IL-3 was dramatic for periods of 1–2 months prior to a crisis when the cultures died out. In 24 cases (42%), cell lines were established; 19 (33%) were dependent on IL-3 for growth and 4 (9%) were factor independent for growth.

The establishment of factor-dependent cell lines was associated with specific tumor types (Table 1). Among the myelogenous leukemias, cells from only one primary tumor failed to grow *in vitro*. Ten of the tumors showed a dependency for IL-3 for growth and eight (67%) were established as cell lines. One factor-independent cell line was obtained from a myelogenous leukemia. Similarly, the erythroleukemias exhibited either limited growth or could be established as cell lines in the presence of IL-3. No factor-independent cell lines were established from the erythroleukemias. Although there were relatively few immunoblastic or follicular center cell lymphomas, the majority were established as factor-dependent cell lines. In contrast, a number of the thymic lymphomas (50%) or lymphoblastic lymphomas (44%) showed no growth *in vitro*. Among those showing growth, few could be established as cell lines. The establishment of factor-independent lines was less correlated with the primary tumor phenotype than was the establishment of factor-dependent cell lines.

Comparison of the Phenotypic Characteristics of Primary Tumors and Cell Lines. There was a good correlation in the phenotypes between the four factor-independent cell lines that were established and the primary tumors from which they were derived (Table 2). Detailed descriptions of these cell lines have been presented elsewhere (16). There was also a good correlation in the expression of Mac-1 between the primary myelogenous leukemias and the factor-dependent cell lines derived from these tumors. The majority of the myelogenous leukemias were Mac-1$^+$ and all the factor-dependent cell lines derived from them were also Mac-1$^+$. The only exception was a Mac-1$^-$ myelogenous leukemia from which a Mac-1$^+$ factor-dependent cell line was derived.

Approximately one-half of the cell lines from myelogenous leukemias also expressed Thy-1. In all but one, both Thy-1$^+$ and Thy-1$^-$ subpopulations existed. In additional experiments (not shown), sorted Thy-1$^+$ and Thy-1$^-$ subpopulations have been shown to give rise to mixed populations,

Table 2. Comparison of phenotypes of primary neoplasms and cell lines

Primary neoplasm			Cell line		
No.	Pathology	Phenotype	No.	Phenotype	IL-3 dependency
9	Myelogenous leukemia	Mac-1$^+$	3	Mac-1$^+$	D
			1	Mac-1$^+$	I
			2	Mac-1$^+$, Thy-1$^{+/-}$	D
			1	Mac-1$^+$, Thy-1$^+$	D
			1	—	D
3	Myelogenous leukemia	—	1	Mac-1$^+$, Thy-1$^+$	D
6	Erythroleukemia	—	3	—	D
16	Thymic lymphoma	Thy-1$^+$	1	Thy-1$^{+/-}$, Mac-1$^{+/-}$	D
1	Thymic lymphoma	Lyb-2$^+$, B220$^+$, ThB$^+$	1	Lyb-2$^+$, B220$^+$, ThB$^+$	I
12	Lymphoblastic lymphoma	Lyb-2$^+$, B220$^+$	2	—	D
			1	Mac-1$^+$, Thy-1$^{+/-}$	D
			1	Lyb-2$^+$, B220$^+$	I
2	Lymphoblastic lymphoma	Lyb-2$^+$	1	Lyb-2$^+$	I
2	Immunoblastic lymphoma	Thy-1$^+$	2	—	D
2	Immunoblastic lymphoma	—	1	—	D
1	Mixed follicular center cell	sIg$^+$, ThB$^+$, I-A$^+$	1	—	D

All tumors were analyzed with the antibodies listed in the *Materials and Methods*; the distinguishing phenotypes are presented; —, no distinguishing phenotype. In all cases, the cells expressed Ly-5, "H-11," Ly-24, and Ly-17.1. B220, Ly-5(B220) as detected by RA3-6B2. Cells were uniformly positive for the antigens listed except when labeled +/−, indicating that both antigen-positive and antigen-negative cells were present. D, dependent for growth; I, independent for growth.

demonstrating that Thy-1 expression is not stable. Recent studies have demonstrated that Thy-1, in addition to T cells, is transiently expressed during myeloid differentiation (19). Consistent with this, the Thy-1^+ cell lines had a myeloid morphology, as noted below.

Primary erythroleukemias were characterized by the absence of the lineage-specific antigens tested for in this study, including Mac-1, Lyb-2, Ly-5 (B220), and Thy-1. The factor-dependent cell lines obtained from erythroleukemias also lacked distinguishing antigens including Mac-1 and Thy-1. Similarly, one of the immunoblastic lymphomas lacked characteristic antigens and gave rise to a factor-dependent cell line with a comparable phenotype. These primary tumors and the cell lines derived from them, however, expressed Ly-17, "H-11," Ly-24, and Ly-5 antigens, which were common to all the tumors and cell lines examined.

As shown in Table 2, there was a less evident correlation between the phenotypes of other primary tumors and the factor-dependent cell lines derived from them. No factor-dependent lines expressed the B-cell-lineage-associated antigens Lyb-2, Ly-5(B220), ThB, or surface immunoglobulin, although three long-term IL-3-dependent lines were derived from Lyb-2^+ Ly-$5(B220)^+$ large pre-B-cell lymphomas. Although a factor-dependent cell line expressing Thy-1 was derived from a thymic lymphoma, phenotypically and morphologically (Fig. 1*h*) the line had myeloid characteristics. As also shown, Thy-1^+ immunoblastic lymphomas gave rise to factor-dependent lines that were Thy-1^-.

The morphological characteristics of several of the cell lines are illustrated in Fig. 1. Most of the lines are myeloid in appearance and have either a ring-shaped nucleus characteristic of early myeloblasts (*c*, *d* and *f*), a heterochromatic nucleus with a large amount of vacuolated cytoplasm, characteristic of monocytes/macrophages (*e*, *g*, *h*, and *i*), or have a pleomorphic nucleus and evidence of cytoplasmic granulation, characteristic of granulocytes (*a*). These lines are morphologically distinct from a factor-independent cell line from a pre-B-cell lymphoma (*b*). There was, however, no morphological distinction between the factor-independent myeloid leukemia cell line (*d*) and some of the factor-dependent myeloid leukemia cell lines (*c* and *f*). Note that although the cell line shown in *c* was uniformly Thy-1^+ and those shown in *g*, *h*, and *i* were heterogeneous for Thy-1 expression, all four had morphological characteristics of myeloid rather than lymphoid cell lineages.

IL-3 Binding of Primary Tumors. For IL-3-dependent cells the percentage of IL-3 binding was proportional to cell number and ≈6.2% of the input IL-3 was bound by 6×10^6

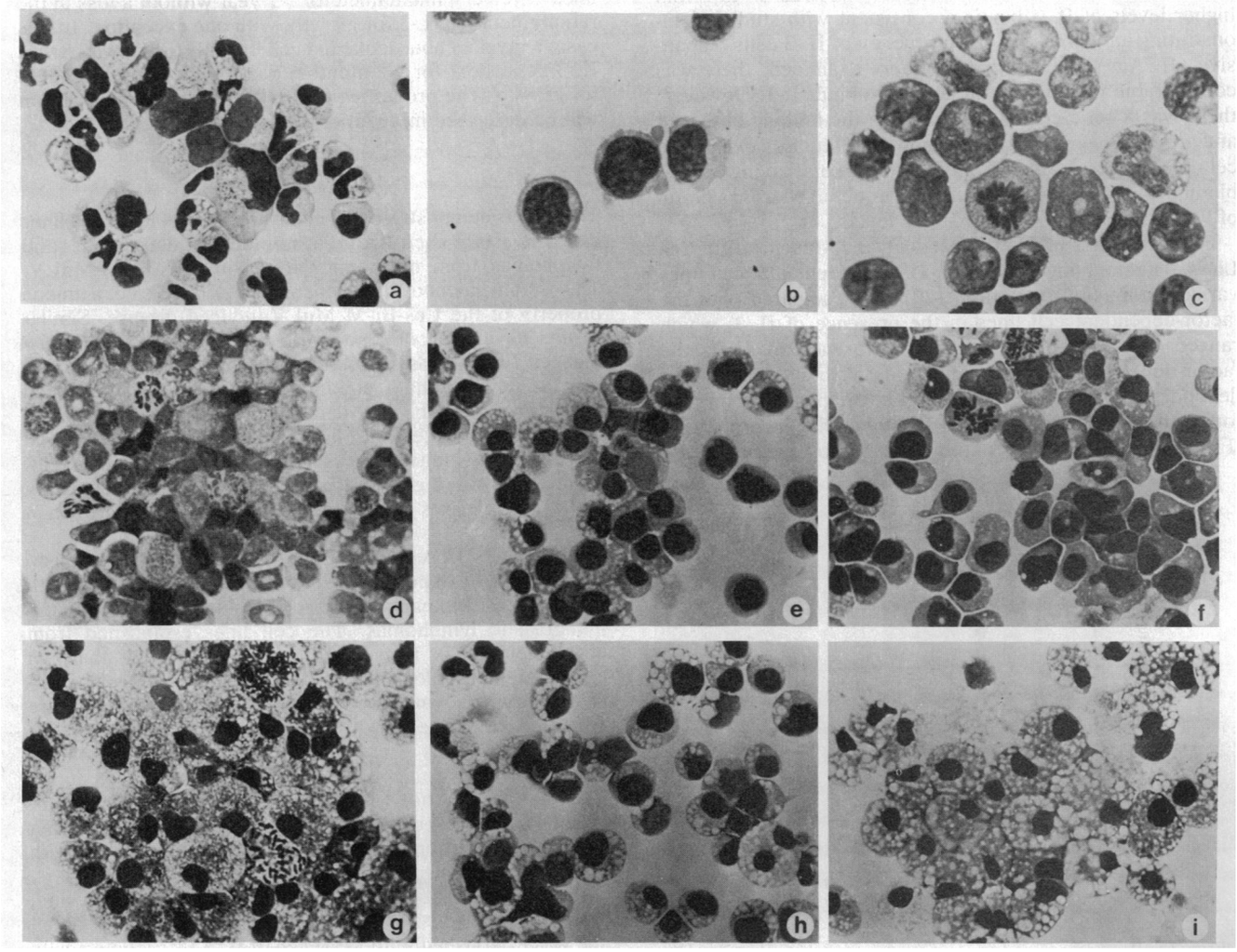

FIG. 1. Cytocentrifuge preparation of cell lines obtained from Cas-Br-M MuLV-induced tumors. The cell lines include an IL-3-dependent line (NFS-36) and a factor-independent cell line (NFS-70) derived from lymphoblastic lymphomas (*a* and *b*, respectively); an IL-3-dependent (NFS-60) and a factor-independent cell line (NFS-124) from two myelogenous leukemias (*c* and *d*, respectively); three IL-3-dependent cell lines from myelogenous leukemias (*e*, NFS- 107; *f*, NFS-78; *i*, NFS-84); IL-3-dependent cell lines from a lymphoblastic lymphoma (*g*, NFS-35); and from a thymic lymphoma (*h*, NFS-22). The preparations were stained with a Wright–Giemsa stain. (×285.)

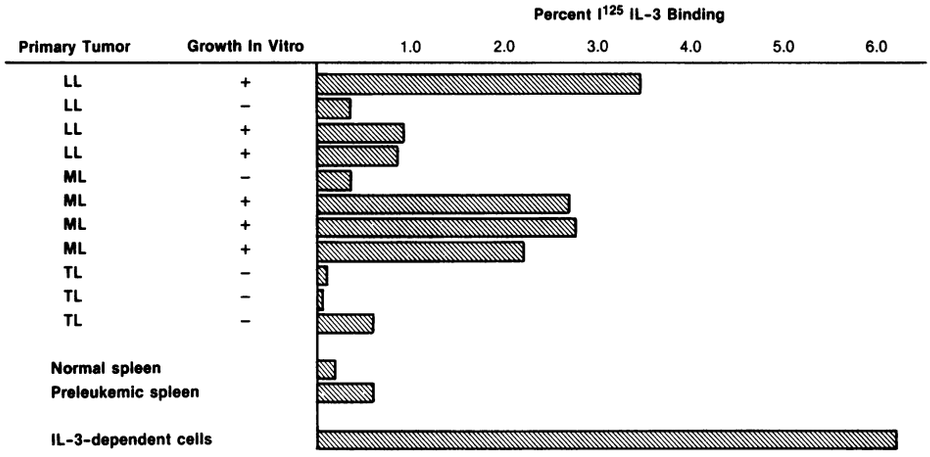

FIG. 2. Binding of ^{125}I-labeled IL-3 by cells from primary tumors, to assess the binding, ≈50,000 cpm with or without a 100-fold excess of unlabeled IL-3 were incubated with 6×10^6 tumor cells for 30 min at 37°C. The extent of cell-bound material was determined as described (18). LL, lymphoblastic lymphoma; ML, myelogenous leukemia; TL, thymic lymphoma. For comparison, the binding levels of an IL-3-dependent cell line from a myelogenous leukemia (NFS-60) is also shown.

cells (Fig. 2). The binding observed with normal spleen cells was generally 0.2%. Spleen cells from virus-inoculated mice, prior to the development of disease, showed 3- to 5-fold higher levels of IL-3 binding, consistent with studies demonstrating an increase in the frequency of IL-3 cells responsive (4). Among the primary tumors examined, there was considerable variation in the ability to bind IL-3. However, there was a good correlation between the binding observed and growth *in vitro* in IL-3. Among the 19 tumors examined, cell lines were obtained from 5 of 10 tumors having >0.6% binding, whereas only one cell line was obtained from a group of 9 tumors having <0.6% binding levels.

Characteristics of IL-3-Dependent and -Independent Cell Lines. The doubling times of the IL-3-independent cell lines varied within the range of 14–20 hr as compared with the factor-dependent cell lines in the presence of IL-3, which ranged from 14 to 36 hr. In the absence of IL-3, the factor-dependent cell lines rapidly lost viability. The factor-dependent cell lines showed an absolute dependence on IL-3 for proliferation as measured by [^{3}H]thymidine incorporation (Fig. 3). Half-maximal proliferation was observed at a concentration of ≈0.2 ng/ml comparable to the concentrations of IL-3 required in other assays (9). The factor-dependent cell lines have been maintained for >1 year without a loss in the requirement for IL-3 for growth, with one exception. In this case, a myelogenous leukemia cell line (NFS-61), which was IL-3 dependent for ≈5 months, became factor independent for growth. The properties of this cell line and its evolution will be described in detail elsewhere.

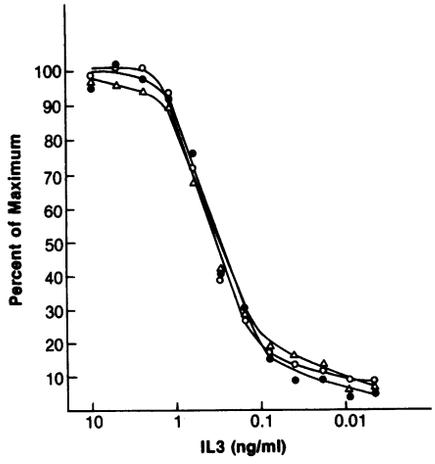

FIG. 3. IL-3 dose–response curves for factor-dependent cell lines. The dose response to purified IL-3 was determined as described (15). Two cell lines obtained from myelogenous leukemias and a cell line obtained from an immunoblastic lymphoma are shown. Comparable results were obtained with all the IL-3-dependent cell lines examined.

DISCUSSION

The results demonstrate that in the presence of IL-3, cell lines can be established from a number of leukemias and lymphomas from mice inoculated with Cas-Br-M-MuLV. The establishment of IL-3-dependent cell lines is not a unique property of the Cas-Br-M MuLV-induced disease. Similar results have been obtained with Moloney leukemia virus-induced lymphomas (1), Rauscher MuLV-induced lymphomas (unpublished data) and Graffi ecotropic MuLV-induced lymphomas (unpublished data). More recently, a series of factor-dependent cell lines has been established from Friend helper MuLV-induced neoplasms using WEHI-3 CM (20). Although purified growth factors were not used, it is likely that these cell lines are also dependent on IL-3 for growth *in vitro*.

The studies presented here demonstrate that the establishment of IL-3-dependent cell lines is associated with myelogenous leukemias, erythroleukemias, and possibly immunoblastic lymphomas. The cell lines established from erythroleukemias possessed a myeloid rather than an erythroid morphology. It is conceivable that both transformed erythroid and myeloid cells exist but that the culture conditions favor the growth of the latter. A second possibility is that a stem cell is transformed and the environment allows one or the other components to predominate. For example, IL-3 has been shown to induce the differentiation of erythropoietin-responsive cells (21), as well as cells that can be induced to differentiate along the myeloid pathway with granulocyte/macrophage colony-stimulating factor (19).

Cell lines could be consistently established from myeloid leukemias in IL-3, whereas only one factor-independent cell line was established in the absence of IL-3. Morphologically and phenotypically, the factor-independent cell lines were similar to the factor-dependent lines. The lack of mature myeloid elements suggests that transformation may involve a block in the ability of the cells to terminally differentiate (19). However, this did not eliminate the requirement for a growth

factor. These results suggest that multiple stages of myeloid leukemias may exist that are associated with changes in the requirement for growth factors, consistent with previous studies (22–24).

The inability to establish cell lines from thymic lymphomas suggests that a factor may also be required for their growth *in vitro*. The normal growth factor requirements for the thymic terminal deoxynucleotidyl transferase-positive cells are not known, although this subpopulation does not proliferate in response to either IL-2 or IL-3 (25). Similarly, the majority of the B-cell lymphomas could not be established as cell lines. In this regard, it is interesting to note that an IL-3-dependent pre-B-cell line has been described (14). Based on this observation, we anticipated some IL-3-dependent lines from the B-cell tumors.

The results suggest that T-cell-derived IL-3 may be required for the maintenance of certain transformed cells *in vivo* in addition to its role in expanding potential target cell populations for transformation. The demonstration that certain leukemias require T-cell-derived growth factors has therapeutic implications. For example, the rapid loss of viability of the myeloid leukemia cell lines in the absence of IL-3 suggests that eliminating the growth factor *in vivo* may have a beneficial effect. Since the production of lymphokines is blocked by cyclosporin A, it will be of interest to assess the effects of this drug on the *in vivo* growth of various types of leukemias.

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1. Ihle, J. N., Rein, A. & Mural, R. (1984) in *Advances in Viral Oncology*, ed. Klein, G. (Raven, New York), Vol. 4, pp. 95–137.
2. Ihle, J. N., Enjuanes, L., Lee, J. C. & Keller, J. (1982) *Curr. Top. Microbiol. Immunol.* **101,** 31–49.
3. Lee, J. C. & Ihle, J. N. (1981) *Nature (London)* **209,** 407–409.
4. Lee, J. C. & Ihle, J. N. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 7712–7716.
5. Ihle, J. N., Pepersack, L. & Rebar, L. (1981) *J. Immunol.* **126,** 2184–2189.
6. Ihle, J. N. & Weinstein, Y. (1984) in *Recognition and Regulation in Cell-Mediated Immunity*, eds. Watson, J. D. & Marbrook, J. (Dekker, New York), in press.
7. Pepersack, L., Lee, J. C., McEwan, R. & Ihle, J. N. (1980) *J. Immunol.* **124,** 279–285.
8. Ihle, J. N., Keller, J., Henderson, L., Klein, F. & Palaszynski, E. W. (1981) *J. Immunol.* **129,** 2431–2436.
9. Ihle, J. N., Keller, J., Oroszlan, S., Henderson, L., Copeland, T., Fitch, F., Prystowsky, M. B., Goldwasser, E., Schrader, J. W., Palaszynski, E., Dy, M. & Lebel, B. (1983) *J. Immunol.* **131,** 282–287.
10. Fung, M. C., Hapel, A. J., Ymer, S., Cohen, D. R., Johnson, R. M., Campbell, H. D. & Young, I. G. (1984) *Nature (London)* **307,** 233–237.
11. Yokota, T., Lee, F., Rennick, D., Hall, C., Arai, N., Mosmann, T., Nabel, G., Cantor, H. & Arai, K. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1070–1074.
12. Ihle, J. N. (1984) in *Contemporary Topics in Molecular Immunology*, eds. Inman, F. P. & Gillis, S. (Plenum, New York), Vol. 10, pp. 93–119.
13. Fredrickson, T. N., Langdon, W. Y., Hoffman, P. M., Hartley, J. W. & Morse, H. C., III (1984) *J. Natl. Cancer Inst.* **72,** 447–454.
14. Palacios, R., Henson, G., Steinmetz, M. & McKearn, J. P. (1984) *Nature (London)* **309,** 126–131.
15. Ihle, J. N., Keller, J., Greenberger, J. S., Henderson, L., Yetter, R. A. & Morse, H. C., III (1982) *J. Immunol.* **129,** 1377–1383.
16. Davidson, W. F., Fredrickson, T. N., Rudikoff, E. K., Coffman, R. L., Hartley, J. W. & Morse, H. C., III (1984) *J. Immunol.* **133,** 744–753.
17. Yeh, C.-J., Hsi, B.-L. & Faulk, W. P. (1981) *J. Immunol. Methods* **43,** 269–275.
18. Palaszynski, E. W. & Ihle, J. N. (1984) *J. Immunol.* **132,** 1872–1878.
19. Ihle, J. N., Morse, H. C., III, Keller, J. & Holmes, K. L. (1984) *Curr. Top. Microbiol. Immunol.* **113,** 86–91.
20. Oliff, A., Oliff, I., Schmidt, B. & Famulari, N. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 5464–5467.
21. Goldwasser, E., Ihle, J. N., Prystowsky, M. B., Rich, I. & Van Zant, G. (1983) *Normal and Neoplastic Hematopoiesis* (Liss, New York), pp. 301–309.
22. Heard, J. M., Fichelson, S., Choppin, J. & Varet, B. (1983) *Int. J. Cancer* **31,** 337–344.
23. Heard, J. M., Fichelson, S., Brigitte, S., Martial, M. A., Berger, R., Varet, B. & Levy, J. P. (1983) *Int. J. Cancer* **32,** 237–245.
24. Heard, J. M., Fichelson, S., Sola, B., Martial, M. A., Varet, B. & Levy, J. P. (1984) *Mol. Cell. Biol.* **4,** 216–220.
25. Keller, J., Weinstein, Y., Hursey, M. & Ihle, J. N. (1985) *J. Immunol.*, in press.