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Specific binding of murine leukemia inhibitory factor to normal and leukemic monocytic cells

(receptor/differentiation induction/clonogenicity/hemopoiesis)

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ABSTRACT **Leukemia inhibitory factor (LIF), a glycoprotein capable of suppressing the clonogenicity and inducing the differentiation of the murine myeloid leukemia cell line M1, was radioiodinated to a high specific radioactivity with retention of full biological activity. Binding of ^{125}I-labeled LIF to M1 cells reached a steady state at 37°C after ≈40 min and was in competition with unlabeled LIF but not granulocyte colony-stimulating factor or a range of other cytokines or differentiation-inducing agents. Specific binding was demonstrable to cells from a range of murine hemopoietic tissues including the bone marrow, the spleen, and the peritoneal cavity. Autoradiography revealed macrophages, monocytes, and their precursors to be the major cell types responsible for ^{125}I-labeled LIF binding within these tissues. Receptors on M1 cells were of high affinity (apparent K_d, 100–200 pM) and few in number (300–500 per cell).**

The progressive expansion of neoplastic populations is not necessarily an irreversible process (1). The demonstration for myeloid leukemias that the naturally occurring hemopoietic regulators, granulocyte colony-stimulating factor (G-CSF) and granulocyte–macrophage colony-stimulating factor (GM-CSF), are able to suppress the clonogenicity and induce the differentiation of some leukemic stem cells (2–4) has raised interest in the possible use of biological regulators as an alternative or adjunct to conventional cytotoxic therapy (5, 6).

Recently, we have purified to homogeneity a glycoprotein termed leukemia inhibitory factor (LIF) that is capable of inhibiting the proliferation and inducing macrophage differentiation of the murine myeloid leukemia cell line M1 (6). Amino acid sequencing (7) and the resultant cloning and sequencing of the cDNA encoding murine LIF (8) demonstrated it to be distinct from other molecules such as G-CSF (9–11), interferon β, interleukin 1 (IL-1), tumor necrosis factor (12), and IL-6 (D.M., unpublished observation) affecting the proliferation and differentiation of M1 leukemic cells. The latter molecules are known to have a variety of actions on normal and neoplastic cells (13–17). To assess the therapeutic potential of LIF, the range of normal and neoplastic cells able to bind or respond to LIF needs to be identified. The present experiments were undertaken to identify cells that might respond to LIF because of their ability to bind ^{125}I-labeled LIF (^{125}I-LIF) specifically and to characterize the interaction of LIF with its receptor on such cells.

MATERIALS AND METHODS

Preparation of Radioiodinated LIF. LIF was purified to homogeneity from Krebs II ascites tumor cell conditioned medium as described (6). The purified material electrophoresed as a single silver-staining protein species with an apparent M_r of 58,000 that comigrated in sodium dodecyl sulfate/polyacrylamide gels with all the detectable M1 differentiation-inducing activity.

Purified LIF (1–2 μg) in 100 μl of 40% (vol/vol) CH_3CN/0.1% (vol/vol) CF_3COOH/2.5 mM $(NH_4)_2CO_3$/0.02% (vol/vol) Tween 20 was radioiodinated by the addition of 2.7 μl (1 mCi; 1 Ci = 37 GBq) of carrier-free Na^{125}I (New England Nuclear) and, while Vortex mixing, 5 μl of 0.2 mM iodine monochloride in 2 M NaCl as described by Contreras *et al.* (18). After 1 min at room temperature, 10 μl of 1 M KI was added and ^{125}I-LIF was separated from unincorporated ^{125}I by sequential gel filtration and cation-exchange chromatography as described (19).

Characterization of ^{125}I-LIF. Both ^{125}I-LIF and unlabeled LIF from the same original fraction were diluted to the same final concentration and assayed in parallel for their capacity to induce the differentiation of M1 cells as described (20). The percentage of radioactivity in the ^{125}I-LIF preparation capable of specifically binding to M1 cells and the specific radioactivity of ^{125}I-LIF were determined by the method of Calvo *et al.* (21).

Binding of ^{125}I-LIF to Cells. Cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum and where required multipotential CSF or GM-CSF and were used when in experimental growth phase. Normal murine hemopoietic cells were obtained from C57BL/6 mice (8–12 weeks old) of either sex.

Binding was carried out as described (19). Cells were resuspended in RPMI 1640 medium containing 10 mM Hepes buffer (pH 7.2) and 10% (vol/vol) fetal calf serum and aliquoted into Falcon 2054 tubes with ^{125}I-LIF and a 20-fold excess of unlabeled LIF or an equivalent volume of medium. After incubation, cell-associated and free radioactivity were separated by centrifugation through fetal calf serum as described (19).

The capacity of cytokines and other agents to compete with ^{125}I-LIF for receptors was tested by preincubating M1 cells with the potential competitor for 60 min on ice before addition of ^{125}I-LIF. Cell autoradiographic analysis was performed as described (19).

RESULTS

Purified ^{125}I-LIF displayed the same specific biological activity in inducing M1 differentiation as unlabeled LIF (Fig. 1*A*). Increasing numbers of M1 cells were incubated with a low level of ^{125}I-LIF in the presence or absence of 100-fold excess unlabeled LIF and the amount of ^{125}I-LIF specifically associated with the cells was determined. By plotting the recip-

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Abbreviations: LIF, leukemia inhibitory factor; ^{125}I-LIF, ^{125}I-labeled LIF; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage CSF; IL, interleukin; MGI-2, macrophage–granulocyte inducer 2.

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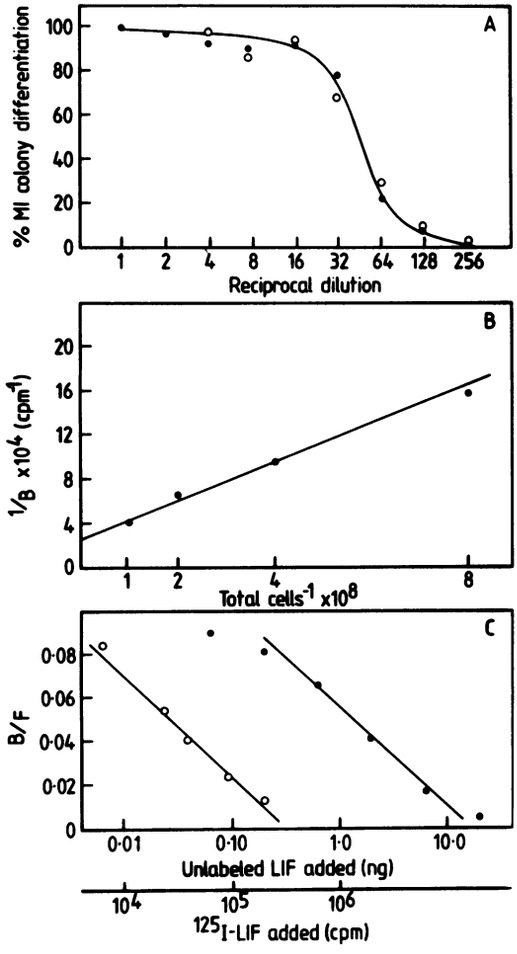

FIG. 1. Radioligand characteristics of the ^{125}I-LIF preparation. (*A*) Comparison of the capacity of labeled (●) and unlabeled LIF (○) to induce the differentiation of M1 colonies grown in agar. (*B*) Measurement of the maximum percentage of ^{125}I-LIF able to bind specifically to M1 cells. ^{125}I-LIF (5000 cpm) was incubated with increasing numbers of M1 cells with or without excess unlabeled LIF, and maximum bindability was determined by extrapolating the plot of reciprocal specifically bound ^{125}I-LIF versus reciprocal cell number to the ordinate (infinite cell number). (*C*) Estimation of specific radioactivity of ^{125}I-LIF. ^{125}I-LIF (11,000 cpm) and either additional ^{125}I-LIF (7000–200,000 cpm) or unlabeled LIF (0.06–20 ng) were incubated with 5×10^6 M1 cells in a total vol of 80 μl for 3 hr on ice. The true free radioactivity was determined by multiplying the total radioactivity (bound + free) by the fraction bindable and subtracting the amount specifically bound. The specific radioactivity was determined by dividing the amount of additional ^{125}I-LIF added by the amount of unlabeled LIF added that yielded the same bound/free (B/F) ratio.

rocal of cell number versus reciprocal ^{125}I-LIF specifically bound (Fig. 1*B*), the maximal amount of ^{125}I-LIF capable of binding specifically was determined from the extrapolated intercept on the ordinate (infinite cell number). In the example shown, the percentage of radioactivity that was bindable ^{125}I-LIF was ≈80% and in different experiments this percentage varied between 70% and 100%.

The specific radioactivity of ^{125}I-LIF was determined by self-displacement analysis. Fig. 1*C* shows that increasing the concentration of LIF, either labeled or unlabeled, resulted in a reduction of the ratio of bound to free ^{125}I-LIF after correction of the free radioactivity by using the bindable fraction determined in Fig. 1*B*. The reduction observed with unlabeled LIF paralleled that of ^{125}I-LIF, suggesting that iodination of the molecule did not alter its capacity to interact with the receptor. It can also be seen from the curves that ^{125}I-LIF displayed, in this case, a specific radioactivity of ≈31,000 cpm/ng. The specific radioactivity for various preparations of radioiodinated LIF varied between 25,000 and 45,000 cpm/ng (0.8 ^{125}I per LIF molecule).

The specificity of ^{125}I-LIF binding was investigated by testing the capacity of various differentiation inducers and growth factors to compete for ^{125}I-LIF binding. Fig. 2*A* demonstrates that unlabeled native LIF could compete for the binding of 90% of the total radioactivity associated with M1 cells. In contrast, other agents capable of inducing M1 differentiation and suppressing M1 clonogenicity, including IL-1α, IL-6, G-CSF, and *Escherichia coli* lipopolysaccharide displayed no capacity to compete with ^{125}I-LIF for binding to M1 cells. Similarly, a range of other agents (Table 1), including IL-1β, IL-2, IL-4, IL-5, multipotential CSF (IL-3), GM-CSF, and macrophage CSF, failed to compete with ^{125}I-LIF for binding to M1 cells.

At 37°C, the amount of ^{125}I-LIF specifically bound to M1 cells increased rapidly, with maximum binding being reached

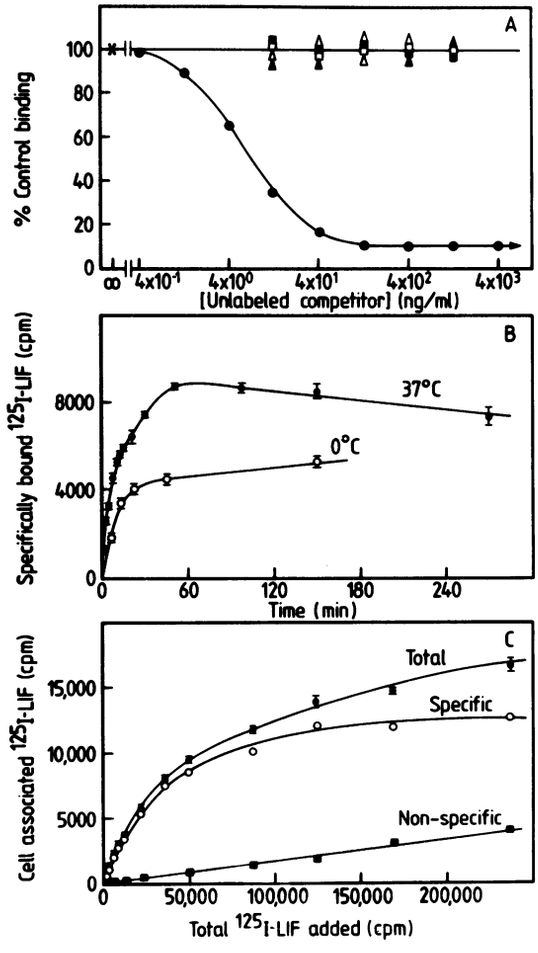

FIG. 2. (*A*) Competition for binding to ^{125}I-LIF receptors on M1 cells. Unlabeled competitors—either LIF (●), G-CSF (□), IL-1 (■), IL-6 (▲), lipopolysaccharide (△), or carrier (*)—were incubated with 10^7 M1 cells on ice for 1 hr before adding 200 pM ^{125}I-LIF and incubating on ice for a further 2 hr. (*B*) Time course of ^{125}I-LIF binding to M1 cells. ^{125}I-LIF (350 pM) with or without excess unlabeled LIF was incubated with M1 cells either at 37°C (●) or on ice (○). After the indicated times, aliquots of the incubation mixture were removed and specifically bound ^{125}I-LIF was determined. (*C*) Saturation analysis of ^{125}I-LIF binding to M1 cells. M1 cells (10^7) were incubated with increasing amounts of ^{125}I-LIF for 40 min at 37°C in a total vol of 80 μl. Total and nonspecific binding were determined by measuring the amount of ^{125}I-LIF bound to M1 cells in the absence or presence of unlabeled LIF, and specific binding was the difference between these values. Total ^{125}I-LIF added (abscissa) was adjusted for the bindability of the ^{125}I-LIF preparation used.

Table 1. Molecular specificity of ^{125}I-LIF binding

Competitor	Concentration, nM	Cell-associated ^{125}I-LIF, cpm
nLIF	2	378 ± 71
rIL-1α	20	3951 ± 21
rIL-1β	20	3875 ± 77
rIL-2	20	3950 ± 230
rIL-4	20	4112 ± 321
rIL-5	20	3971 ± 86
nIL-6	20	3999 ± 102
rMulti-CSF	200	3913 ± 206
rGM-CSF	200	3988 ± 41
rG-CSF	200	4013 ± 108
nM-CSF	100	4070 ± 126
Saline		3913 ± 185

Binding was on 5 × 10^6 M1 cells in 100 μl of binding medium. Cells were preincubated with competitor on ice for 1 hr, after which ^{125}I-LIF (100 pM) was added and incubation was continued for a further 3 hr on ice. n, Native purified molecules; r, recombinant; M-CSF, macrophage CSF; Multi-CSF, multipotential CSF. Mean and range of duplicate determinations is shown.

after ≈40 min. In contrast, the maximum level of binding reached on ice was lower (4800 cpm versus 8500 cpm) and the approach to steady state was slower than at 37°C (Fig. 2*B*).

Cells from normal murine hemopoietic tissues and a range of murine cell lines were tested for their capacity to bind ^{125}I-LIF specifically. In the results shown in Table 2, binding of various cells is expressed relative to that displayed by an equivalent number of C57BL/6 bone marrow cells tested in parallel. Specific binding ranged from 3.7-fold greater than bone marrow for resident peritoneal cells to 0.66-fold and 0.10-fold that of bone marrow for spleen cells and thymus cells, respectively.

The capacity of LIF to bind specifically to murine cell lines was tested. Table 2 demonstrates that M1 bound ≈11-fold more LIF than C57 bone marrow cells. Two other macrophage/myeloid cell lines, HA-15 and AC-5, were also positive, while the remaining hemopoietic cell lines, including WEHI-3B D$^+$, WEHI-3B D$^-$, NFS-60, NFS-61, AC-8, WR19, WEHI-265, J774, P388D1, P-815, 416B, FDCP-1, 32D clone 3, 32D clone 13, T59, LB-3, E9D4, and EL-4, and the nonhemopoietic cell lines, L929 and Krebs II, bound no detectable ^{125}I-LIF specifically.

Table 2. Cellular specificity of ^{125}I-LIF binding

Cell type (description)	Specific ^{125}I-LIF binding
Positive cell types	
Normal hemopoietic cells	
Bone marrow	1.00 ± 0.14
Spleen	0.66 ± 0.11
Thymus	0.10 ± 0.03
Resident peritoneal cells	3.71 ± 0.86
Cell lines	
M1 (monocytic leukemia)	10.85 ± 1.12
HA-15 (macrophage leukemia)	0.99 ± 0.30
AC-5 (myeloid leukemia)	0.76 ± 0.14
Negative cell lines	
Cell lines	
WEHI-3B D$^+$ (myelomonocytic leukemia)	0.00 ± 0.03
J774 (macrophage nonleukemic)	0.27 ± 0.30
P388D1 (macrophage nonleukemic)	−0.14 ± 0.06
LB-3 (T lymphoid)	0.03 ± 0.04
L929 (fibroblast)	−0.27 ± 0.28

Binding was to 5 × 10^6 cells in a total vol of 80 μl with ^{125}I-LIF at 200 pM (with or without 20-fold excess unlabeled LIF) at 37°C for 40 min. Specific binding is expressed as the ratio of that seen on an equivalent number of C57BL bone marrow cells included in each experiment. The mean and range of two to four separate experiments with duplicate points with and without LIF are shown.

Binding of ^{125}I-LIF to M1 cells as a function of increasing ^{125}I-LIF added is shown in Fig. 2*C*. Nonspecific binding was linear with the amount of ^{125}I-LIF added over the concentration range tested while specific binding was saturable and the isotherm was hyperbolic in shape. Similar results were observed for a range of cell types tested including bone marrow, spleen, and peritoneal cells. However, for the cell lines WEHI-3B D$^+$, NFS-61, and 32D clones, no specific binding was detected over the same ^{125}I-LIF concentration range.

Scatchard analyses of typical saturation curves for M1 and WEHI-3B D$^+$ cells are shown in Fig. 3*A*. In several such experiments, a single receptor site of high apparent affinity (apparent K_d, 100–200 pM) and of relatively low frequency (350–500 per cell) was evident on M1 cells; however, no specific binding was detectable on WEHI-3B D$^+$ cells. Similar Scatchard plots were observed for lipopolysaccharide-induced peritoneal cells and spleen cells (Fig. 3*B*), in which a single class of receptor was present with apparent dissociation constants of 100 and 75 pM and average receptor frequencies of 70 and 14 per cell, respectively.

Cell autoradiography (Fig. 4) indicated there was heterogeneity in the capacity of M1 cells to bind LIF and that the major cell type binding ^{125}I-LIF in peritoneal and spleen cell preparations was monocyte/macrophage. Similarly, in the bone marrow, monocytes, macrophages, and their precur-

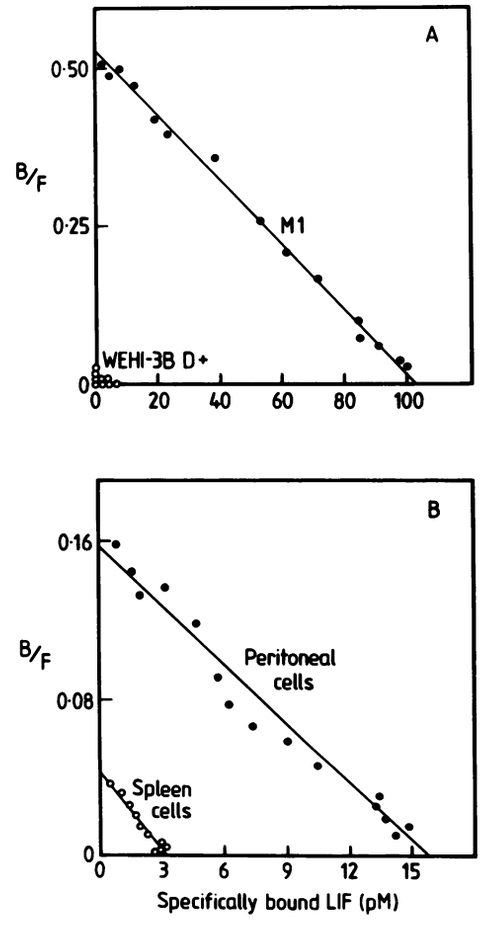

FIG. 3. Scatchard analysis of ^{125}I-LIF saturation isotherms. Cells were incubated with increasing amounts of ^{125}I-LIF as described in the legend to Fig. 2*C*. Specifically bound (B) and free (F) ^{125}I-LIF were determined and adjusted to the corresponding values for 10^7 cells. Scatchard plots (*A*) for ^{125}I-LIF on M1 cells (●), WEHI-3B D$^+$ (○) and (*B*) for peritoneal cells (●) and spleen cells (○).

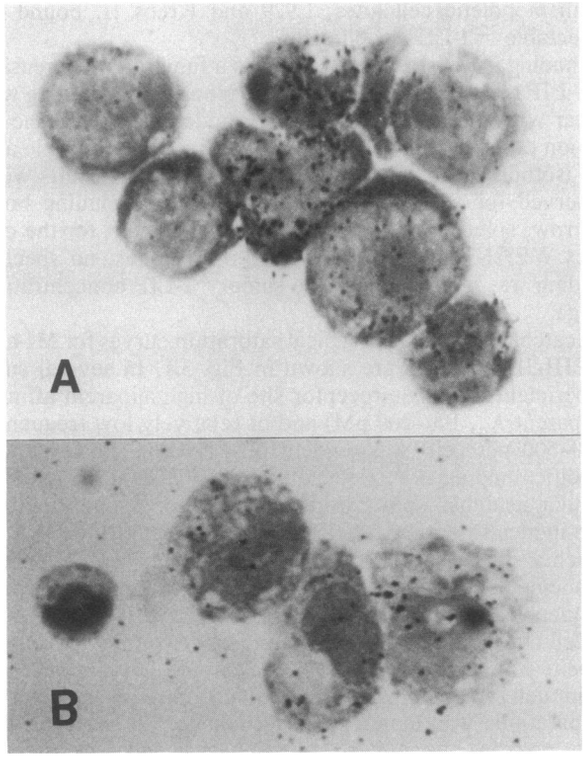

FIG. 4. Autoradiographs of ^{125}I-LIF binding to (*A*) M1 myeloid leukemic cells showing heterogeneity of labeling and (*B*) resident peritoneal cells showing labeling selectively on monocytic cells.

sors were the most obvious cells binding LIF (data not shown). No binding was observed to cells of the neutrophilic or eosinophilic granulocyte lineages, mast cells, erythrocytes, and a large majority of lymphocyte-like cells. The observation that relatively few cells in spleen cell populations specifically bound ^{125}I-LIF accounted for the low calculated average receptor number on spleen cells compared with peritoneal and M1 cells.

DISCUSSION

^{125}I-LIF bound specifically to cells of the leukemic line M1, which differentiate to macrophage-like cells in response to LIF (20). More interestingly, with respect to elucidating the physiological function of LIF, specific binding of LIF was also observed to normal macrophages, monocytes, and their precursors. This suggests that LIF may play a role in the generation and/or functional regulation of cells of the macrophage lineage. While macrophages and monocytes in normal hemopoietic tissues bound LIF, of the 18 immortalized or leukemic myeloid cell lines tested, only M1 cells bound high levels of LIF specifically. It remains to be established whether cells displaying LIF receptors are selected against in the generation of myeloid cell lines or whether such cell lines, once established, lose their LIF receptors during *in vitro* passage.

The interaction of LIF with its receptor, like that of the other myeloid-specific regulators macrophage CSF, GM-CSF, and G-CSF (22–24), is of high affinity, with an apparent K_d of 80–200 pM. Half-maximal induction of M1 differentiation occurs at a concentration of LIF capable of occupying only ≈5% of receptors; that is, between 15 and 25 of the 350–500 receptors on a single cell.

A variety of physiological and nonphysiological agents have been shown to induce M1 differentiation (25, 26). Several such agents, including G-CSF, IL-1, IL-6, and lipopolysaccharide, showed no capacity to compete directly with ^{125}I-LIF for receptor binding sites under conditions in which unlabeled LIF competed for 85–95% of ^{125}I-LIF binding. This suggests that LIF acts via a specific cell surface receptor in its action on M1 cells.

Two other factors with functional and some structural similarity to LIF have been described. The first of these, termed differentiation factor (D-factor), has been purified from several sources (27, 28) and radioiodinated (29). ^{125}I-labeled D-factor bound specifically to M1 cells with an apparent affinity and receptor number comparable to LIF. The binding of the second factor, macrophage–granulocyte inducer 2 (MGI-2), has been investigated by utilizing an adsorption protocol in which cells were allowed to bind impure MGI-2 on ice. Bound and free MGI-2 were then separated and bound MGI-2 was eluted under acidic conditions and subsequently quantitated by a biological assay (30). Using this method, Lotem and Sachs claimed that M1, WEHI-3B, peritoneal macrophages, and peritoneal neutrophils expressed 1200–3000 MGI-2 receptors per cell with an affinity of ≈1 nM. These results differ markedly from those obtained with ^{125}I-LIF but are difficult to interpret because nonspecific binding of MGI-2 could not be quantitated and the biological assay used to quantitate MGI-2 would not distinguish between MGI-2, G-CSF, IL-1, and IL-6, all of which could be present in the crude source of MGI-2.

The present results have indicated that LIF induces differentiation through a receptor not recognized by other cytokines or agents known to induce differentiation of the M1 myeloid leukemic cell line, such as G-CSF. This suggests that differentiation can be accomplished by separate mechanisms and, indeed, there is evidence for synergy in the action of G-CSF and LIF on M1 cells (20). The description of LIF receptors on normal murine monocytes and macrophages also suggests that, like G-CSF, LIF could have a role in the control of normal hemopoiesis.

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1. Metcalf, D. (1983) *Pathology* **15**, 1–3.
2. Metcalf, D. (1983) *Leuk. Res.* **7**, 117–132.
3. Metcalf, D. (1979) *Int. J. Cancer* **24**, 616–623.
4. Nicola, N. A. & Metcalf, D. (1981) *J. Cell. Physiol.* **109**, 253–264.
5. Metcalf, D. (1987) *Proc. R. Soc. London Ser. B* **230**, 389–423.
6. Hilton, D. J., Nicola, N. A. & Metcalf, D. (1988) *Anal. Biochem.*, in press.
7. Simpson, R. J., Hilton, D. J., Nice, E. C., Rubira, M. R., Metcalf, D., Gearing, D. P., Gough, N. M. & Nicola, N. A. (1988) *Eur. J. Biochem.*, in press.
8. Gearing, D. P., Gough, N. M., King, J. A., Hilton, D. J., Nicola, N. A., Simpson, R. J., Nice, E. C., Kelso, A. & Metcalf, D. (1987) *EMBO J.* **6**, 3995–4002.
9. Tomida, M., Yamamoto-Yamaguchi, Y., Hozumi, M., Okabe, T. & Takaka, F. (1986) *FEBS Lett.* **207**, 271–275.
10. Tsuda, H., Neckers, L. M. & Pluznik, D. H. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4317–4321.

11. Hilton, D. J., Nicola, N. A., Gough, N. M. & Metcalf, D. (1988) *J. Biol. Chem.*, in press.
12. Onozaki, K., Urawa, H., Tamatani, T., Iwamura, Y., Hashimoto, T., Baba, T., Suzuki, H., Yamada, M., Yamamoto, S., Oppenheim, J. J. & Matsushima, K. (1988) *J. Immunol.* **140,** 112–119.
13. Nicola, N. A. (1987) *Int. J. Cell. Cloning* **5,** 1–15.
14. Symons, J. A., Dickens, E. M., Di Giovine, F. & Duff, G. N. (1984) in *Lymphokines and Interferons*, eds. Clemens, M. J., Norris, A. G. & Gearing, A. J. H. (IRL, Oxford), pp. 269–289.
15. Oppenheim, J. J., Matsushima, K., Yoshimura, T. & Leonard, E. J. (1985) *Immunol. Lett.* **16,** 179–184.
16. Old, L. J. (1985) *Science* **230,** 630–632.
17. Kanano, M., Hirano, T., Matsuda, T., Taga, T., Horii, Y., Swato, K., Asaoku, H., Tang, B., Tanabe, O., Tanaka, H., Kuramoto, A. & Kishimoto, T. (1988) *Nature (London)* **332,** 83–85.
18. Contreras, M. A., Bale, N. F. & Spar, I. L. (1983) *Methods Enzymol.* **92,** 277–292.
19. Nicola, N. A. & Metcalf, D. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 3765–3769.
20. Metcalf, D., Hilton, D. J. & Nicola, N. A. (1988) *Leukemia* **2,** 216–221.
21. Calvo, J. C., Radicella, J. P. & Charreau, E. H. (1983) *Biochem. J.* **212,** 259–264.
22. Guilbert, L. J. & Stanley, E. R. (1980) *J. Cell Biol.* **85,** 153–159.
23. Nicola, N. A. & Metcalf, D. (1985) *J. Cell. Physiol.* **124,** 313–321.
24. Walker, F. & Burgess, A. W. (1985) *EMBO J.* **4,** 933–939.
25. Hozumi, M. (1985) *CRC Rev. Hematol. Oncol.* **3,** 235–277.
26. Sachs, L. (1978) *Nature (London)* **274,** 535–539.
27. Tomida, M., Yamamoto-Yamaguchi, Y. & Hozumi, M. (1984) *J. Biol. Chem.* **259,** 10978–10982.
28. Tomida, M., Yamamoto-Yamaguchi, Y. & Hozumi, M. (1984) *FEBS Lett.* **178,** 291–296.
29. Yamamoto-Yamaguchi, Y., Tomida, M. & Hozumi, M. (1988) *Exp. Cell Res.* **164,** 97–102.
30. Lotem, J. & Sachs, L. (1987) *Int. J. Cancer* **40,** 532–539.