Interleukin-3 and Granulocyte-Monocyte Colony-Stimulating Factor Receptors on Human Acute Myelocytic Leukemia Cells and Relationship to the Proliferative Response

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Interleukin-3 (IL-3) and granulocyte-monocyte-colony-stimulating factor (GM-CSF) stimulate proliferation of human acute myeloid leukemia (AML) in vitro, although patterns of response among clinical cases are diverse. Whether regulatory abnormalities related to growth factor responses in human AML may establish the outgrowth of the neoplasm is unclear. We determined receptor numbers and affinity for IL-3 and GM-CSF on human AML cells using human recombinant IL-3 (rIL-3) and GM-CSF (rGM-CSF). In 13 of 15 cases of primary AML high-affinity (kd 26 to 414 pmol/L) receptors for IL-3 were demonstrable on the cells. The average numbers of IL-3 receptors ranged from 21 to 145 receptors per cell. Normal WBCs showed IL-3 receptors on their surface at similar densities. IL-3 receptor positivity often correlated with GM-CSF receptor positivity of AML; GM-CSF receptors were demonstrated on the cells of 11 of 15 cases, although average numbers of GM-CSF receptors were ten times greater. The in vitro response of the cells to exogenous IL-3 or GM-CSF was examined by measuring thymidine uptake. Because IL-3 and GM-CSF were potent inducers of DNA synthesis in vitro, apparently relatively few receptors are required to permit activation of growth. These experiments did not provide evidence for overexpression or increased receptor sensitivity as an explanation for AML growth. In a minority of cases, however, the cells were unable to respond to IL-3 (four of 15 cases) or GM-CSF (four of 15 cases) despite normal receptor availability on the cell surface.

INTERLEUKIN-3 (IL-3) and granulocyte-monocyte-colony-stimulating factor (GM-CSF) belong to the class of CSFs which regulate survival, proliferation, and differentiation of hematopoietic progenitor cells in humans.[1-4] Most cases of human acute myelocytic leukemia (AML) are dependent on CSF for growth, but some leukemias appear to proliferate spontaneously as the consequence of autocrine CSF production.[5-8] The growth pattern of AML cells cultured in vitro with different CSFs shows considerable variation.[9-12] However, regulatory abnormalities of growth have not been established in human AML since this diverse growth reactivity may resemble the heterogeneity of response of different stages of normal marrow precursor cells rather than cellular alterations as the result of malignant transformation. CSFs are believed to react with their target cells through membrane-bound receptors.[13,14] Little is known about the role played by growth factor receptors in the pathophysiology of human AML. The relationship between growth factor receptor expression and stimulability of AML cells in vitro has not been investigated. Overexpression of a receptor may have a role in the outgrowth of AML in vivo, but no experimental evidence to support this possibility has yet been provided. One study reported that GM-CSF receptor density on AML cells is not increased; hence, overexpression of GM-CSF receptors apparently does not account for a leukemic growth advantage.[15] IL-3 is also an effective growth factor for blast cells of AML,[9-12] but no information about IL-3 receptor properties on human AML cells is available. For a better understanding of the mechanisms that lead to proliferation of AML cells in response to IL-3 and GM-CSF, we investigated numbers and affinity of IL-3 and GM-CSF receptors on cells of patients with AML and correlated these data with the proliferative response of the cells to both molecules.

MATERIALS AND METHODS

Preparation of cells. AML cells were obtained from 15 cases of AML classified according to the criteria of the French-American-British (FAB) committee[16,17]: M1 (n = 2), M2 (n = 3), M4 (n = 8), and M5 (n = 2). The cells were separated from bone marrow (BM) or peripheral blood (PB) after bovine serum albumin (BSA) density-gradient Ficoll-Isopaque centrifugation[18] and subsequent removal of E rosette-forming cells. The AML cells were cryopreserved in 7.5% dimethyl sulfoxide (DMSO) and 20% inactivated fetal calf serum (FCS). The viability of the cells after thawing was always >95%. Normal BM was obtained by posterior iliac crest puncture from healthy adults, and the mononuclear cells were separated over Ficoll-Isopaque density gradient. Eosinophilic cells were separated from the blood of a patient with hypereosinophilia (80×10^9 cells/L) and WBCs from the blood of healthy subjects after sedimentation in 0.1% methyl cellulose. The latter cell preparations were used fresh.

Hematopoietic growth factors. rIL-3 (*Escherichia coli* derived)[19] and rGM-CSF [Chinese hamster ovary (CHO) cell derived][20] was prepared and purified at Genetics Institute (Cambridge, MA). GM-CSF was glycosylated and had a molecular weight (mol wt) of 20 to 30 Kd (biologic activity 6 to 12×10^9 U/g). IL-3 was not glycosylated (mol wt 15 Kd; 5 to 10×10^9 U/g).

Radioiodination of IL-3 and GM-CSF. Purified rIL-3 and GM-CSF were radiolabeled according to the method of Bolton and Hunter.[21] Bolton-Hunter reagent (BHR) 1 mCi (Amersham Laboratories, Amersham, England) dissolved in benzene was dried under a stream of N_2 in the packing vial at 22°C. The vial was then cooled to 0°C, and 5 μg rIL-3 or 5 μg rGM-CSF in 20-μL borate buffer (0.1 mol/L pH 8.2) was applied. The reaction was permitted to continue for 45 minutes at 0°C while the vials were agitated every five minutes. Labeling was quenched by adding 100 μL glycine (0.2 mol/L in borate buffer). After 5 minutes on ice, 100 μL 0.25% gelatin was added to facilitate recovery of the reactant from the vial.

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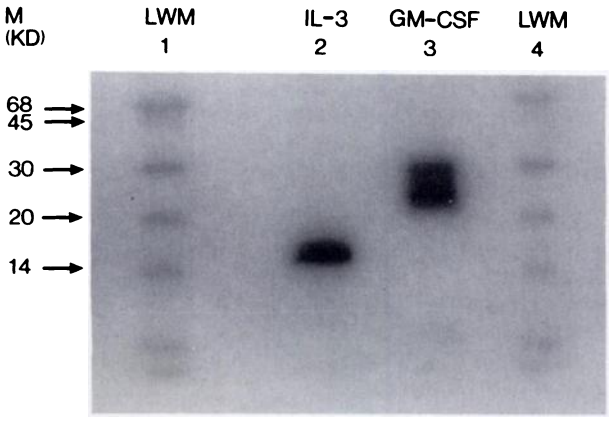


Fig 1. Autoradiography of ^{125}I-labeled IL-3 and GM-CSF. Radioiodinated IL-3 (lane 2) and GM-CSF (lane 3) 4 × 10^5 cpm were electrophoresed in 12% SDS polyacrylamide gel. Autoradiograms were obtained by exposing the gel to Kodak X-Omat AR film (Kodak, Rochester, NY) at −70°C. Lanes 1 and 4: iodine-labeled mol-wt markers.

The labeled proteins were separated from the unreacted BHR and the iodinated glycine by chromatography on sepharose columns (GF-5, Pierce Chemical Co, Rockford, IL) equilibrated with phosphate-buffered saline (PBS) containing 0.1% gelatin and 0.01% Tween 20.

Samples taken from the void volume were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent autoradiography showing a single band for IL-3 at 15 Kd and a broader band for GM-CSF at 20 to 30 Kd (Fig 1). Radiolabeling of IL-3 and GM-CSF by the Bolton and Hunter protocol resulted in preparations that retained their ability to stimulate colony formation by normal BM cells in methylcellulose (Table 1). The reduced effectiveness in stimulating colony formation by higher concentrations of radiolabeled IL-3 and GM-CSF is most likely attributed to the continuous irradiation by iodine 125 throughout culturing time (14 days). TCA precipitation showed <5% nonprecipitable radioactivity for both factors. The maximum binding capacity was estimated at 35% to 50% for IL-3 and 40% to 70% for GM-CSF. This given was used to correct the "free" cpm in the Scatchard calculations. Specific activity of labeled IL-3 and GM-CSF was determined by self-displacement analysis[22] or calculated from the trichloroacetic acid (TCA)-precipitable radioactivity measured in a small sample taken from the reactant (similar results were obtained with both methods, although the self-displacement for IL-3 was somewhat more inaccurate as compared with the TCA method). The specific radioactivity was estimated at 8 to 10 × 10^4 cpm/ng for IL-3 and 4 to 6 × 10^4 cpm/ng for GM-CSF. The fractions containing the radiolabeled protein were stored at 4°C in 20% glycerol and 0.2% sodium azide and used within 4 weeks of preparation.

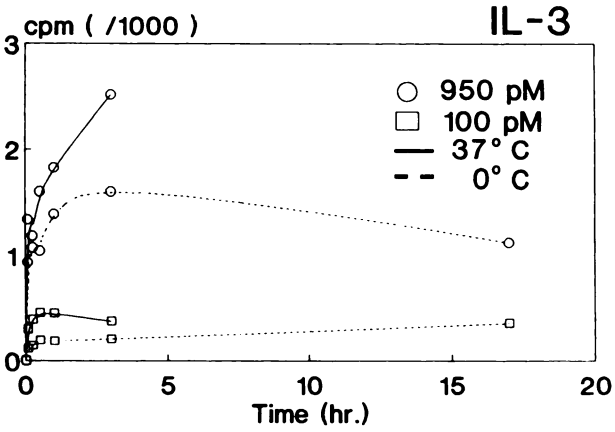
Table 1. Biologic Activity of Radioiodinated IL-3 and GM-CSF

	No. of Colonies/Clusters		
CSF Concentration (pmol/L)	100%	Mixture 50%/50%	^{125}I 100%
IL-3			
0.3	0/7	0/6	0/2
3	6/21	5/28	3/18
30	14/39	15/41	5/28
300	11/30	0/0	0/0
GM-CSF			
2	2/7	3/5	0/6
20	12/34	22/38	36/35
200	31/31	37/43	29/40
2000	52/41	20/47	0/8

Dose-response of titrated concentrations of native IL-3, radiolabeled IL-3, and a mixture of native/radiolabeled IL-3 and the same dose-response data for GM-CSF. Colonies and clusters induced from 0.5 × 10^5 Ficoll-separated marrow cells in 1-mL cultures were scored at day 14. Values indicate average numbers of colonies/clusters of duplicate cultures.

Colony culture assay. The biologic activity of radiolabeled IL-3 and GM-CSF was determined in normal BM: 0.5 × 10^5 cells were cultured in a 1-mL mixture of Iscove's modified Dulbecco's medium (IMDM), 0.8% methylcellulose, 30% FCS, BSA, transferrin, lecithin, sodium-selenite, and 2-mercaptoethanol.[23] Nonlabeled and radiolabeled IL-3 and GM-CSF were added to the cultures in tenfold-increasing concentrations. The experiment also included cultures containing a 50%/50% mixture of nonlabeled and radiolabeled CSF to establish the negative effects of γ radiation on colony formation. Clusters >15 cells and colonies >50 cells were scored at day 14. Duplicate cultures were established for each point.

Binding of radiolabeled IL-3 and GM-CSF to AML cells. After thawing, AML cells were washed twice in Hanks' balanced salt



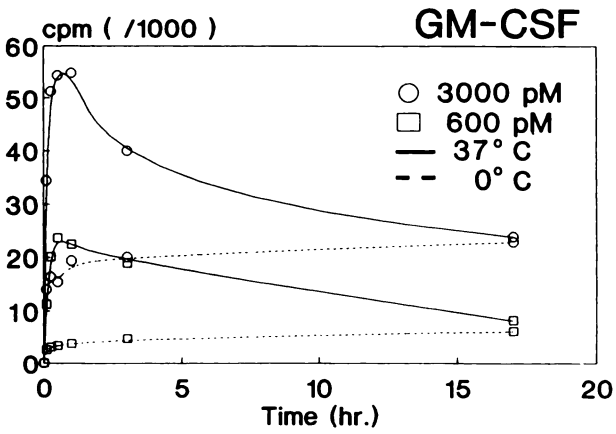


Fig 2. Binding of ^{125}I-labeled IL-3 and GM-CSF to normal WBCs in relation to time. WBCs 4 × 10^6 were incubated with 98 and 950 pmol/L IL-3 or 600 and 3,000 pmol/L GM-CSF at 0 and 37°C. Binding was assessed as a function of time of incubation.

solution (HBSS). Usually, 2 to 4 × 10^6 cells were incubated for one hour at 37°C in 100 μL α minimal essential medium (α-MEM)/10% FCS with 10 to 2,000 pmol/L radiolabeled IL-3 or GM-CSF with or without 100 nmol/L nonlabeled factor. The cells were then cooled to 0°C, layered over 500 μl calf serum in Eppendorff tubes on ice and centrifuged for five minutes at 1,000 g. The tubes were frozen in liquid nitrogen, and the tips were cut off for counting in a Packard γ-counter. Specific binding was defined as the difference between the amount of radioactivity bound without unlabeled factor added, and the amount of radioactivity bound with excess unlabeled factor added. Experiments were conducted in duplicate. Receptor numbers and binding affinities were derived by Scatchard analysis.[24]

^{3}H-Thymidine incorporation into AML cells. DNA synthesis of AML cells was measured as described.[25] Cells 2 × 10^4 were cultured for three days in 96-well round-bottom microtiter trays in 100 μL serum-free medium, with or without addition of IL-3 or GM-CSF. Four hours before harvesting, 0.1 μCi tritiated thymidine (Amersham) was added. Cells were harvested on nitrocellulose paper with a Titertek Harvester 550 (Flow Laboratories, Isrike, UK). Radioactivity was determined with a scintillation counter (Beckman LS 3800, Fullerton, CA). All experiments were performed in triplicate and data are mean dpm. In each experiment, preirradiated cells were used in control cultures to measure background thymidine uptake.

RESULTS

Binding of ^{125}I IL-3 and ^{125}I GM-CSF to human WBCs and eosinophils. The binding kinetics of ^{125}I IL-3 and ^{125}I GM-CSF to human WBCs reveal that binding is more effective at 37°C than at 0°C (Fig 2). After one-hour exposure at 37°C, the amount of cell-associated radioactivity began to decrease. Specific binding of IL-3 and GM-CSF at 37°C was then determined for WBCs (Fig 3A and B); binding of IL-3 to eosinophils was also determined (Fig 3C). Scatchard plots indicated relatively low numbers of IL-3 receptors (134 sites/cell) of single-class affinity (kd 113 pmol/L) on WBCs. Eosinophils expressed more receptors (1,950 sites/cell) with a kd of 494 pmol/L. Comparative IL-3 and GM-CSF receptor data for human WBCs of four

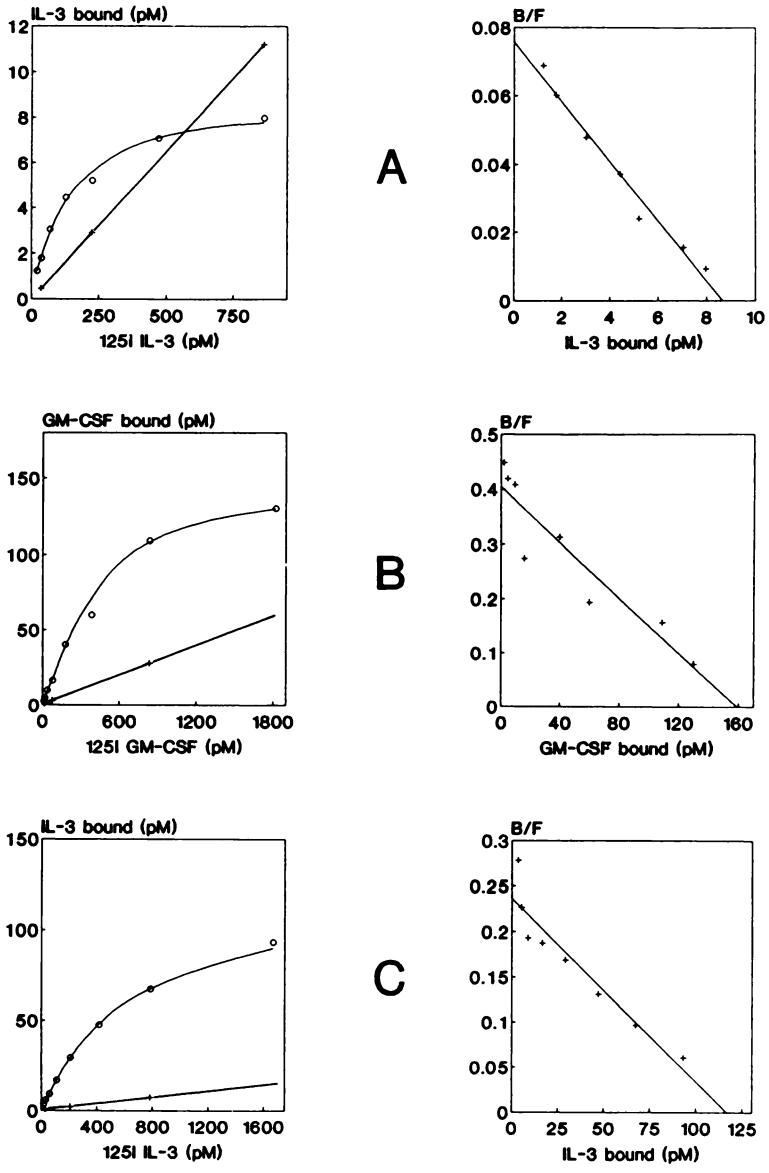


Fig 3. Binding of radiolabeled IL-3 (A) and GM-CSF (B) to WBCs and IL-3 to eosinophils (C). Cells 2 to 5 × 10^6 were incubated with increasing amounts of radiolabeled IL-3 or GM-CSF for one hour at 37°C. Nonspecific binding was determined in the presence of excess unlabeled factor. Specific binding (o) as well as nonspecific binding (+) data are plotted (left panels). Each point is the mean of two estimates. Scatchard plots of these data (right panels).

Table 2. Binding of ^{125}I IL-3 and ^{125}I GM-CSF to WBCs and Eosinophils

Cell Type	IL-3 Receptors		GM-CSF Receptors	
	Sites/Cell	kd (pmol/L)	Sites/Cell	kd (pmol/L)
WBCs				
Donor 1	137	86	1,579	230
Donor 2	60	53	1,789	392
Donor 3	74	82	1,515	497
Donor 4	134	113	2,091	493
Eosinophils	1,950	494	1,991	636

Receptor numbers (mean per cell) and kd were derived from binding experiments and subsequent analysis according to Scatchard (described in legend to Fig 3). WBCs were obtained from different donors.

donors and the patient with eosinophilia are shown in Table 2.

Binding of ^{125}I IL-3 and ^{125}I GM-CSF to AML cells. In 13 of 15 cases, receptors for IL-3 could be demonstrated on AML cells (Fig 4A and Table 3). The average density of IL-3 receptors varied between 21 and 145 receptors per cell. The kd ranged from 26 to 414 pmol/L, which is in the same order of magnitude as the kd of IL-3 receptors of normal WBCs (68 to 113 pmol/L). In 11 of 15 cases, both IL-3 receptors and GM-CSF receptors were expressed on the cells (Table 3). The mean numbers of GM-CSF receptors ranged from 23 to 1,263 receptors per cell (kd 64 to 404 pmol/L). Thus, the average numbers of GM-CSF receptors per cell were approximately ten times greater than those of IL-3 receptors. GM-CSF receptor number on cells of patients with AML of FAB classification M1 or M2 tended to be less than those of patients of the M4 and M5 categories. In contrast, IL-3 receptor expression on the cells was not different between AML cases of different FAB subtypes.

Table 3. Binding of Human Radioiodinated IL-3 and GM-CSF to Their Receptors on AML Cells

FAB Classification	Case No.	Blasts* (%)	IL-3 Receptors		GM-CSF Receptors	
			Sites/Cell	kd (pmol/L)	Sites/Cell	kd (pmol/L)
M1	1	98	56	91	23	64
	2	100	25	467	< Det.	—
M2	3	100	21	326	< Det.	—
	4	ND	< Det.	—	40	157
	5	97	55	176	84	123
M4	6	83	17	183	236	157
	7	89	33	414	561	293
	8	82	21	137	191	162
	9	64	< Det.	—	843	404
	10	85	60	61	245	155
	11	ND	51	65	792	258
	12	ND	38	26	550	197
	13	ND	98	39	783	169
M5	14	67	113	163	949	169
	15	50	145	88	1263	274

Abbreviations: < Det., below detection level; ND, not determined.

Binding assays were performed in duplicate after incubation of 2 to 5 × 10^6 AML cells for one hour at 37°C with eight different concentrations of radiolabeled IL-3 or GM-CSF. Mean receptor numbers per cell and kd were derived from Scatchard plots (Fig 4).

*Blast percentage of AML cells used in binding experiments.

Proliferation of AML cells in vitro: Comparison with IL-3 and GM-CSF receptor expression. The stimulative effects of human IL-3 and GM-CSF on DNA synthesis of AML

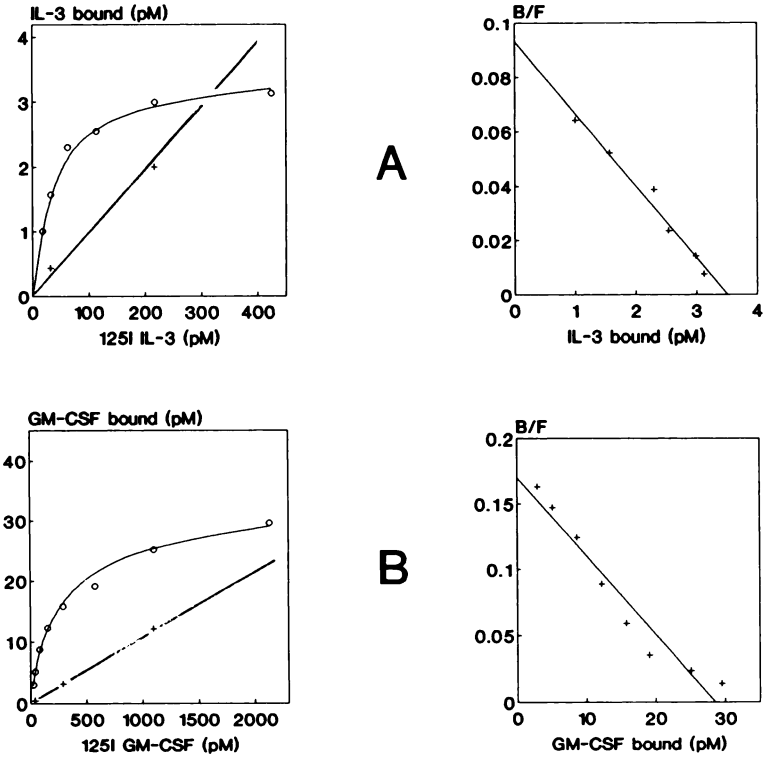


Fig 4. Binding of radiolabeled IL-3 (A) and GM-CSF (B) to AML cells (patient 13) was performed as described in the legend to Fig 3.

Table 4. Relationship Between Stimulability of ^{3}H-TdR Uptake by IL-3 and IL-3 Receptor Positivity of AML Cells

FAB	Case No.	^{3}H-TdR Uptake (dpm x 10^{-2}) (IL-3 Added) No	200 U/mL	IL-3 Responder	IL-3 Receptors*
M1	1	4.5	15.1	+	+
	2	1.2	1.6	–	+
M2	3	0.7	0.8	–	+
	4	3.6	8.8	–	–
	5	31.0	90.0	+	+
M4	6	19.1	57.5	+	+
	7	11.1	20.2	+	+
	8	39.2	88.9	+	+
	9	14.5	41.5	+	–
	10	11.9	92.0	+	+
	11	53.6	54.6	–	+
	12	90.0	89.4	–	+
	13	80.8	122.4	+	+
M5	14	11.4	26.9	+	+
	15	3.3	17.4	+	+

^{3}H-TdR incorporation by AML cells was determined in triplicate cultures with or without IL-3. When mean dpm of stimulated cultures was significantly greater than the values of unstimulated control cultures, IL-3 responses were considered positive (+). AML cases are classified according to FAB cytology.

*IL-3 receptor positivity based on data shown in Table 3.

cells in vitro were determined and compared with the growth factor receptor data (Tables 4 and 5). IL-3 was able to increase tritiated thymidine uptake of AML cells in ten of 15 cases (Table 4). In nine of the ten IL-3 responders, receptors for IL-3 could be demonstrated, whereas in one IL-3 responder (patient 4) no specific binding of IL-3 was measurable. Probably in the latter patient, the cells expressed IL-3 receptors below the sensitivity of the assay. Of the five nonresponders, in four instances IL-3 receptors were demonstrated. In all GM-CSF–responsive patients (9 of 15), GM-CSF receptors were demonstrable on the cells (Table 5). Among the six nonresponders, GM-CSF receptors were not detectable in two patients, whereas significant numbers of GM-CSF receptors were demonstrated on the cells of the four remaining nonresponsive patients.

Table 5. Relationship Between Stimulability of ^{3}H-TdR Uptake by GM-CSF and GM-CSF Receptor Positivity of AML Cells

FAB	Case No.	^{3}H-TdR Uptake (dpm x 10^{-2}) (GM-CSF Added) No	200 U/mL	GM-CSF Responder	GM-CSF Receptors
M1	1	4.5	10.2	+	+
	2	1.2	1.1	–	–
M2	3	0.7	0.5	–	–
	4	3.6	6.4	–	+
	5	31.0	91.0	+	+
M4	6	19.1	38.9	+	+
	7	11.1	23.2	+	+
	8	39.2	78.4	+	+
	9	14.5	26.3	+	+
	10	11.9	32.2	+	+
	11	53.6	55.2	–	+
	12	90.0	91.3	–	+
	13	80.8	89.0	+*	+
M5	14	11.4	13.0	+*	+
	15	3.3	6.8	–	+

Details as in Table 4.

*We considered inhibition of spontaneous proliferation by anti-GM-CSF evidence of autocrine stimulation and scored those cases as GM-CSF responders as well.

DISCUSSION

GM-CSF receptors have been demonstrated on AML cells at 44 to 1,074 sites per cell with a kd of 17 to 290 pmol/L using glycosylated GM-CSF[15] and up to 40 sites per cell (kd 10 to 68 pmol/L) using nonglycosylated GM-CSF.[26] The expression of IL-3 receptors on AML cells has not yet been investigated. The present studies of 15 patients with AML were undertaken to assess IL-3 receptors on AML cells as well as the proliferative response of the cells to IL-3 and to relate these data to GM-CSF receptor expression and stimulability.

High-affinity IL-3 receptors are apparent on the cells of most cases of AML (13 of 15). The density of the IL-3 receptors is relatively low (23 to 145 sites per cell). In one of the two IL-3 receptor-negative cases (patient 9) a proliferative response could be evoked by IL-3, which suggested that these cells carried IL-3 receptors at a density below the sensitivity of the assay. Apparently, the number of receptors necessary to elicit a proliferative response in AML cells may be minimal. IL-3 and GM-CSF receptors were often coexpressed on the cells of the same cases of AML. This observation is of interest since apparent similarities exist between the spectra of stimulative abilities of these two growth factors.[27]

Notably, density as well as affinity of the IL-3 receptors of AML cells are on the same order as those of normal human WBCs; kd and mean numbers of IL-3 receptors per cell did not vary as a function of the cytologic classification of the AML cells. In contrast, the more immature AML types expressed fewer GM-CSF receptors than those of M4 and M5 cases as well as those of WBCs. Although the limited number of cases does not permit firm conclusions regarding the relationship of receptor density and FAB subtype of AML, GM-CSF receptor density may increase as a function of progressive maturation, a phenomenon previously reported by other investigators as well.[15] In the one patient with hypereosinophilia, IL-3 receptors were expressed at higher levels (2,208 sites per cell). This observation is compatible with autoradiography data in mice, indicating that eosinophils express high numbers of IL-3 receptors.[28]

Demonstration of IL-3 receptors did not always appear to be predictive of the proliferative response of the cells to IL-3 (Table 4). In five patients, DNA synthesis could not be induced with IL-3. Because in one nonresponder IL-3 receptors were not demonstrated, a true absence of IL-3 receptors may have determined the lack of response. The cells of the other four nonresponders however, expressed average numbers of IL-3 receptors, comparable to those of the responding

cases of AML. Thus, in those four patients, IL-3 was incapable of inducing DNA synthesis although IL-3 receptors were available on the cells at normal values. The same phenomenon was observed for GM-CSF; ie, in four of the six patients whose cells did not respond to GM-CSF (patients 4, 11, 12, and 15), we could demonstrate GM-CSF receptors on the cells. The explanation for this discrepancy between positive growth factor receptor expression and negative response is elusive. These cells may already have been activated by autocrine CSF production, obscuring a measurable IL-3 or GM-CSF response. Indeed, in two nonresponders expressing IL-3 as well as GM-CSF receptors (patients 11 and 12) the cells showed high spontaneous activity. In neither case could the spontaneous proliferation be blocked by neutralizing anti–IL-3 or anti–GM-CSF antibodies. Therefore, these experiments did not provide evidence for endogenous IL-3 or GM-CSF stimulation, although growth of these cells due to autocrine stimulation by IL-3/GM-CSF inside the cells may have masked exogenous stimulation by IL-3 or GM-CSF. However, in certain other patients IL-3 (patients 2 and 3) and GM-CSF nonresponders (patients 4 and 15) with positive IL-3/GM-CSF receptor expression, the cells did not proliferate spontaneously. Why AML cells of those patients expressed IL-3 or GM-CSF receptors but were incapable of responding to these factors is thus unclear. The cells of these patients may have been deficient beyond the level of receptor binding, which would not allow them to elicit a normal proliferative response. Investigations focusing on signal transduction after stimulation with CSFs may be necessary to clarify this question.

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