Jpn. J. Cancer Res. 80, 887–894, September 1989

Binding Properties and Proliferative Effects of Human Recombinant Granulocyte-Macrophage Colony-stimulating Factor in Primary Leukemia and Lymphoma

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Binding of radiolabeled human granulocyte-macrophage colony-stimulating factor (GM-CSF) was studied with blast cells from eight patients with acute myeloblastic leukemia (AML), and neoplastic lymphoid cells from one patient with acute lymphoblastic leukemia (ALL), two patients with chronic lymphocytic leukemia (CLL) and one patient with undiagnosed B cell neoplasia. In all AML cases studied, Scatchard graphs of the direct binding data were curvilinear, and were best fitted by curves derived from a two-binding-site model; one site with high affinity (Kd_1=12–71 p*M*; 174–602 sites/cell) and the other with low affinity (Kd_2=0.5–2.7 n*M*; 1137–6020 sites/cell). A cross-linking study on blast cells from one AML patient demonstrated specific bands which were similar to those reported for peripheral blood neutrophils. Furthermore, blast colony assays for the same preparations showed remarkable proliferative response to GM-CSF in the concentration range from 0.3 n*M* to 7.0 n*M* (ED_{50}>0.7 n*M*). This concentration range is approximately one order of magnitude higher than that which is effective for colony formation from normal bone marrow progenitors (ED_{50}≒0.1 n*M*). No significant correlation could be observed between the responsiveness of blast progenitors to GM-CSF, and the numbers or affinities of GM-CSF binding sites demonstrated on blast cells. In studies with neoplastic lymphoid cells from four patients, ^{125}I-GM-CSF also specifically bound in two cases, while response to GM-CSF was not observed in these cases. These results indicate that the expression of GM-CSF receptor is not restricted to the GM-CSF-responsive AML blast cells, but can be observed in other AML blast cells and even in neoplastic lymphoid cells.

Key words: Blast colony — GM-CSF receptor — Acute leukemia — Lymphoid neoplasia

Among a wide range of biological activities of human granulocyte-macrophage colony-stimulating factor (GM-CSF[4]),[1] proliferative effects on blast cells from patients with acute myeloblastic leukemia (AML) have been reported by several authors.[2, 3] Receptors for this factor were also demonstrated on normal and leukemic hematopoietic cells using ^{125}I-labeled recombinant GM-CSF.[4–7] Recent papers described a single class of high-affinity binding site for peripheral blood neutrophils, various human myeloid cell lines[4, 5] and blast cells from patients with AML.[6] In contrast, we have obtained data suggesting the existence of two classes of GM-CSF binding site on two GM-CSF-responsive cell lines and peripheral blood monocytes, and demonstrated three separate bands labeled by ^{125}I-GM-CSF in a cross-linking study.[7] In the present study, we examined the binding of bacterially synthesized ^{125}I-labeled human GM-CSF to blast cells from patients with AML, and the blast colony assay was carried out using the same cell preparations. Next, we studied ^{125}I-GM-CSF binding and response to GM-CSF for neoplastic cells from patients with lymphoid malignancies, since recent reports have disclosed that a murine T-cell line was stimulated by murine GM-CSF,[8, 9] ^{125}I-labeled murine GM-CSF specifically bound to some T-cell lines[10] and even human nonhematopoietic cells responded to human GM-CSF.[11, 12]

MATERIALS AND METHODS

Cell preparation Heparinized peripheral blood was collected from eight patients with AML at diagnosis or relapse, and from patients with acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL), after obtaining informed consent. Heparinized pleural fluid was collected from one patient with undiagnosed B cell neoplasia, again after obtaining informed consent. Hematological data of these patients are shown in Table I. Diagnosis was made according to the French-American-British (FAB) classification[13] for acute leuke-

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[4] The abbreviations used in this paper are: GM-CSF, granulocyte-macrophage colony-stimulating factor; BSA, bovine serum albumin; DSS, disuccinimidyl suberate; FCS, fetal calf serum; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; rh, recombinant human; AML, acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia.

mia. CLL was diagnosed on the basis of morphology and surface marker analysis. A patient presented as case 12 in Table I was disclosed to have a significant amount of pleural fluid containing numerous monotonous, large, round cells. The results of surface marker and Southern blot analyses of these cells revealed monoclonal proliferation of B cells. Neither lymph-node enlargement nor abnormal mass was detected in spite of extensive examination. Mononuclear cells were separated from peripheral blood or pleural fluid of these patients by centrifugation through a Ficoll-Hypaque density gradient (1.077 g/ml). Depletion of T lymphocytes by rosette formation with sheep erythrocytes was performed for AML cases, and depletion of adherent cells with a macrophage-separating plate (MSP; Jimro, Japan)[14] was performed for cases with lymphoid malignancies. Wright-Giemsa staining disclosed that each of these preparations contained more than 95% morphologically homogeneous cells. These cells were used immediately for both binding and colony assay, or stored in liquid nitrogen in α-minimal essential medium (α-MEM, GIBCO, Grand Island, NY) with 50% fetal calf serum (FCS, GIBCO) and 10% dimethylsulfoxide until use.

Heparinized peripheral blood was obtained from normal volunteers. Adherent cells and phagocytic cells were sequentially removed as described previously.[14, 15] Further depletion of T lymphocytes was performed by rosette formation with sheep erythrocytes. T lymphocytes were obtained as the nonadherent population after passage of mononuclear cells through a nylon-wool column (Fenwal Laboratories, CA) as described previously.[16]

Recombinant human GM-CSF (rhGM-CSF) Bacterially synthesized human GM-CSF[17] (10^8 U/mg protein by bone marrow colony assay, purity >99%) was kindly provided by Sumitomo Pharmaceutical Co., Ltd., Osaka.

Iodination of rhGM-CSF Radiolabeling by the method of Bolton and Hunter[18] with minor modification was performed as described elsewhere.[7] Briefly, 1 mCi of Bolton-Hunter reagent was evaporated with a gentle stream of N_2 and incubated with 5 μg of GM-CSF in 10 μl of phosphate buffer (pH 8.0) for 12–16 h at 4°C. The reaction mixture was applied to a Sephadex G-25 column (Pharmacia, Uppsala, Sweden) equilibrated with phosphate-buffered saline without magnesium and calcium (PBS(−), Flow Laboratories) containing 0.1% gelatine and 0.02% sodium azide, eluted with the same buffer and stored in the presence of 0.1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO) at 4°C. This preparation showed specific radioactivity of $4–6\times10^4$ cpm/ng, and its binding capacity was stable for at least 6 weeks. The specific radioactivities were determined by self-displacement analysis, corrected for maximal binding capacity.[7, 19] Biological activities were fully retained in the growth stimulation assay of GM-CSF-dependent human cell line TF-1.[7, 20]

Binding of ^{125}I-GM-CSF to leukemic cells T lymphocyte-depleted myeloid leukemic cells, adherent cell-depleted neoplastic lymphoid cells and normal peripheral blood lymphocytes were washed twice with binding buffer (RPMI 1640 medium (GIBCO) supplemented with 0.1% BSA, 25 m*M* 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes, Sigma; pH 7.4), 0.01% bacitracin (Wako Pure Chemicals, Osaka), and 0.02% NaN_3 at 4°C. Cells (2.5×10^6 in 200 μl of binding buffer) containing various concentrations of ^{125}I-GM-CSF with or without a 50-fold excess of unlabeled GM-CSF were incubated for 90 min at 15°C with frequent gentle agitation. The separation of bound from free radioactivities was performed by centrifugation on a phthalate oil.[7] Specific binding was defined as the difference between the amount of radioactivity bound in the absence and presence of unlabeled GM-CSF. For Scatchard analysis,[21] the parameters were calculated by computer using the least-squares fitting program, "SALS."[22]

Cross-linking study Affinity labeling of the GM-CSF receptor was performed using a homobifunctional chemical cross-linking reagent, disuccinimidyl suberate (DSS, Pierce Chemical Co., Rockford, IL), as previously described.[7] Briefly, 5×10^6 cells were incubated with ^{125}I-GM-CSF (5 n*M*) in the presence or absence of 100-fold excess of unlabeled GM-CSF. The washed cell pellets were resuspended in ice-cold 500 μl of PBS(−) followed by addition of DSS at a final concentration of 0.2 m*M* and incubation on ice for 15 min. The cell extract was analyzed by SDS-PAGE according to Laemmli.[23]

Blast colony assay The effects of rhGM-CSF on blast colony formation were tested as described by Hoang *et al.*[2] with minor modifications. Briefly, cells were incubated in 24 multi-well dishes (Falcon, NJ) in 0.5 ml of α-MEM supplemented with 0.8% methylcellulose (Dow Chemicals, Midland, MI) and 20% FCS, with various concentrations of GM-CSF. After 7 days' incubation at 37°C in a humidified atmosphere of 5% CO_2 in air, colonies of more than 20 cells were scored under an inverted microscope. Four wells were used for each assay, and the mean ± SD was determined.

RESULTS

Scatchard analysis of ^{125}I-GM-CSF binding to AML blast cells As shown in Table I, ^{125}I-GM-CSF specifically bound to blast cells in all AML cases studied. Figure 1 shows saturation binding studies followed by Scatchard analysis for blast cells of four representative AML patients out of eight, in which the concentrations of incubated ^{125}I-GM-CSF were varied from 15 p*M* to 5 n*M*. Scatchard graphs were curvilinear, and were best fitted

by curves derived from a two-binding-site model. Binding parameters of the hypothetical two sites on the AML blast cells' surfaces were Kd_1=12–71 p*M*, 174–602 sites/cell and Kd_2=0.5–2.7 n*M*, 1137–6020 sites/cell. Essentially the same results were obtained in the remaining four AML cases.

Cross-linking study for AML blast cells We performed a cross-linking study for blast cells from an AML patient (M4) (not included in Table I) to estimate the molecular masses of the GM-CSF receptors. The percentage of blast cells was greater than 95% based on wright-Giemsa staining and surface marker analysis, and the blast colony assay with these cells demonstrated weak stimulation by GM-CSF. As shown in Fig. 2, specific bands were detected under reducing conditions, and disappeared on incubation with a 100-fold excess of unlabeled GM-CSF. Although each band was poorly separated from the others, the molecular masses of these bands were similar to those detected for myeloid leukemic cell lines and peripheral blood neutrophils (150, 115 and 95 kDa) as described elsewhere.[7]

Blast colony assay for AML blast cells Figure 3 shows the effects of rhGM-CSF on blast colony formation assayed in methylcellulose culture. In AML, blast colonies were formed in the cases of patients 1, 2, 4 and 5 in the absence of GM-CSF. GM-CSF stimulated the growth of blast progenitors from all but two patients in a dose-dependent fashion. The response to GM-CSF was heterogeneous among the patients. In patients 1, 3 and 6, GM-CSF stimulated the blast colony formation from zero or a very small number of colonies to 300–500 colonies at maximum. In patients 4 and 5, the number of colonies/10^5 cells was increased from 500 to 800. A weak stimulatory effect of GM-CSF was observed in patient 7, and no stimulatory effect was detected in patients 2 and 8. In all GM-CSF-responsive cases, the number of colonies increased dose-dependently over 0.7 n*M* GM-CSF, up to 7 n*M*. Morphology of the colony-forming cells evaluated by Wright-Gimmsa staining was the same as that of primary blast cells. These results are summarized in Table I. No correlation was apparent between the numbers of GM-CSF binding sites and the response to GM-CSF of blast progenitors.

^{125}I-GM-CSF binding to lymphoid cells Binding of ^{125}I-GM-CSF was also studied for neoplastic cells from patients with lymphoid malignancies. Interestingly, cells from two out of four patients showed specific binding to ^{125}I-GM-CSF. Scatchard curves for these cells were also biphasically curvilinear (Fig. 4), and were best fitted by curves derived from a two-site model, suggesting that two classes of GM-CSF binding site could be expressed on certain neoplastic cells of lymphoid lineage. To study

Table I. Patients' Characteristics and Binding Parameters of ^{125}I-GM-CSF to Blast Cells and Neoplastic Lymphoid Cells from These Patients

Patient	Diagnosis	WBC ($\times 10^{-3}$ /mm^3)	Blasts in WBC (%)	High affinity		Low affinity		Response in colony assay
				Kd (p*M*)	Receptor number	*Kd* (n*M*)	Receptor number	
1	M1	16.5	90	51	250	0.7	1137	+++
2	M1	1.2	95	43	180	0.9	1565	−
3	M1	40.0	90	17	240	2.7	3973	+++
4	M2	80.0	78	71	602	1.5	3621	++
5	M3	94.0	95	14	190	2.2	3901	++
6	M4	33.6	90	13	174	0.5	1324	+++
7	M5b	52.3	62	50	602	1.1	6020	+
8	RAEB-T ↓ AML	13.3	94	12	277	0.6	3010	−
9	ALL[a]	238.0	95	11	40	1.7	722	−
10	CLL[a]	69.7	93[c]	29	60	1.6	482	−
11	CLL[a]	23.0	90[c]	No specific binding				−
12	B-T[a]	10.0[b]	99[c]					−

a) Surface markers observed were as follows; patient 9, HLA-DR 90%, CALLA 86%; patient 10, B1, 94%, IgD 77%; patient 11, B4 82%, B1 94%, IgM 85%; patient 12, B4 98%, IgM 93%. Patient 12 had an undiagnosed B cell-origin tumor. See "Materials and Methods" for details.
b) Cells harvested from pleural fluid.
c) Lymphoid cells instead of blasts.

whether normal lymphocytes have receptors for GM-CSF, we also tested ^{125}I-GM-CSF binding to various fractions from peripheral blood mononuclear cells in normal subjects (Fig. 5). The specific binding of ^{125}I-GM-CSF was decreased in proportion to the degree of monocyte deprivation from peripheral blood mononuclear cells. The fraction No. 4 in Fig. 5 was expected to contain mainly B lymphocytes, and the amount of specific binding to this fraction could be due to contaminating monocytes. We further examined GM-CSF binding to T lymphocytes fractionated on a nylon-wool column. This fraction, which contained few contaminating monocytes, showed no specific binding (data not shown). These experiments suggested that normal B or T lymphocytes have very few or no receptors for GM-CSF.

Blast colony assays for neoplastic lymphoid cells The effects of GM-CSF on blast colony formation were tested for the neoplastic cells from lymphoid malignancies. The results, as summarized in Table I, were rather different from those in AML cases; quite a large number of colonies was observed in one case (case 12) without addition of GM-CSF, but GM-CSF did not affect the colony formation in this case. In the other three cases, no colonies were formed in the presence or absence of GM-CSF. Again, it was found that the cells with GM-CSF-binding sites did not respond to GM-CSF stimulation under the conditions we used.

DISCUSSION

We have recently obtained data suggesting the existence of two classes of binding site for GM-CSF in monocytes and GM-CSF-responsive cell lines, and we proposed the multi-chain model for human GM-CSF

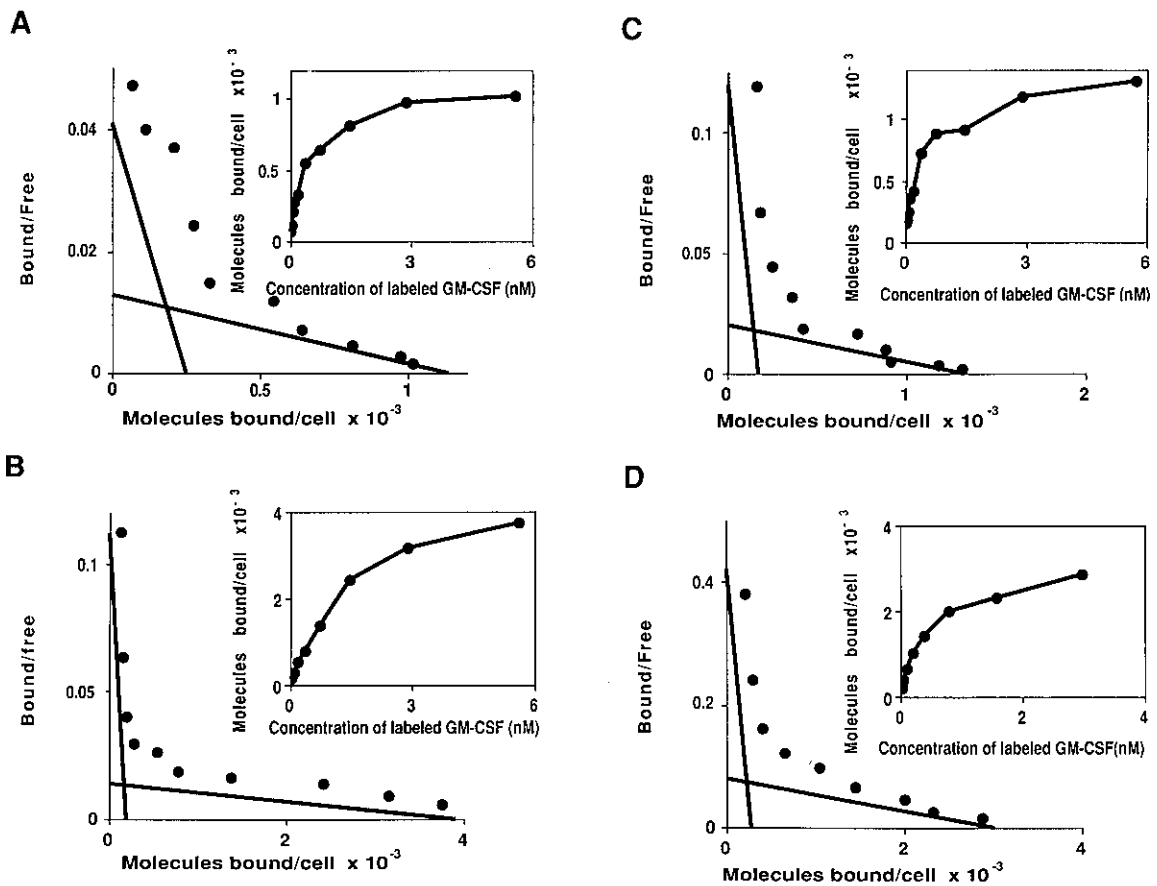

Fig. 1. Scatchard analysis of ^{125}I-GM-CSF binding to AML blasts. Cells (1×10^6/200 μl binding buffer) were incubated with various concentrations of ^{125}I-GM-CSF in the presence or absence of an excess of unlabeled GM-CSF. The figure shows representative Scatchard plots of binding data for four out of eight patients; Case 1 (A), Case 5 (B), Case 6 (C) and Case 8 (D). Binding parameters are summarized in Table I.

receptor.[7] However, it seems reasonable that the receptors with lower affinity (dissociation constant of approximately 1–2 n*M*) do not mediate any functions, since all the biological actions exerted through GM-CSF receptor were produced at concentrations in the range of 1–100 p*M* in the previous reports.[5] Recently, some investigators showed that recombinant GM-CSF was an active growth factor for primary AML blast cells.[2,3] They reported, in addition, that the growth response of AML blasts to GM-CSF was quite heterogeneous among the patients. We have observed that recombinant GM-CSF stimulated the colony formation by AML blasts at much higher concentrations in some patients than those required for maximal colony formation from normal progenitor cells (unpublished data). Kelleher *et al.* also obtained an exponential curve of colony-formation by AML blasts at one order of magnitude higher concentrations than those for normal bone marrow mononuclear cells.[6]

These results prompted us to study the GM-CSF receptor on AML blasts in order to determine whether the receptors expressed on primary AML blasts are identical to those on normal cells, and whether correlations are apparent between the response of AML blasts to GM-CSF and the number and affinity of receptors for GM-CSF. Kelleher *et al.*[6] reported a single class of GM-CSF binding sites for AML blast cells, but they did not refer to the correlation between the response, and affinities or numbers of GM-CSF receptors.

Scatchard plots of ^{125}I-GM-CSF binding to all the AML blast cells were curvilinear, indicating that the binding interactions were complex. Interactions between identical binding sites (insulin receptor)[24] or different binding affinities with different subunits of the receptor

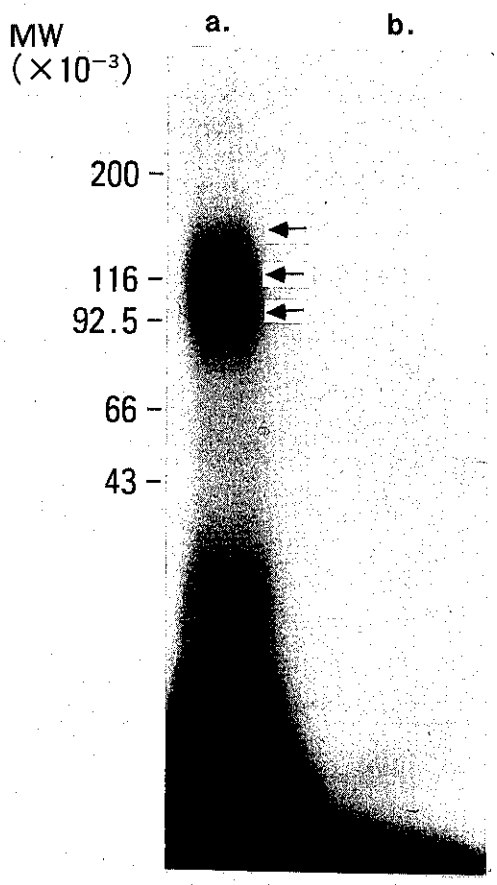

Fig. 2. Cross-linking of ^{125}I-GM-CSF to AML blasts. Cells (5×10^6/500 μl binding buffer) were incubated with ^{125}I-GM-CSF (approximately 3 n*M*) in the presence (b) or the absence (a) of a 100-fold excess of unlabeled GM-CSF. Specific bands, which disappeared on incubation with an excess of unlabeled GM-CSF, were detected.

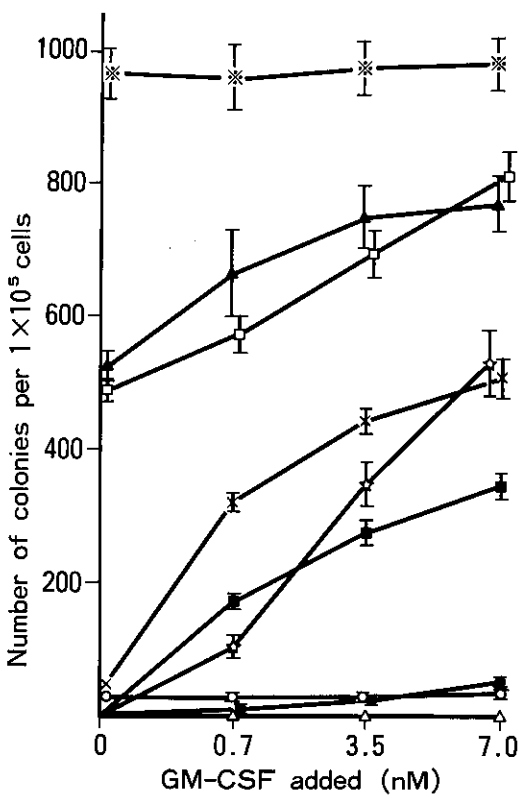

Fig. 3. Clonal proliferation of blast cells. Colony formation from 1×10^5 leukemic blast cells in the presence of various concentrations of GM-CSF is shown. Six out of eight AML patients responded to GM-CSF. In all GM-CSF-responsive cases, the number of colonies increased dependently on GM-CSF dose up to 7 n*M*. Neoplastic cells from patients with lymphoid malignancies did not respond to GM-CSF. Patient 1, ×; 2, ○; 3, ☆; 4, □; 5, ▲; 6, ■; 7, ●; 8, 9, 10, 12, △; and 11, ※.

(interleukin 2 receptor)[25] resulted in curvilinear Scatchard plots. With the GM-CSF receptor system, we have suggested the involvement of two different molecules with different binding affinities on the basis of a cross-linking experiment using U-937 cells.[7] Therefore, a two-binding-site model seems to be realistic in the GM-CSF receptor system, and it is possible that a low-affinity binding site exists as well as a high-affinity one for all the AML blast cells tested in the present study.

We looked for a correlation between the dissociation constants or numbers of both the high- and low-affinity binding sites, and the response of AML blasts. However, no correlation was observed between these parameters. Binding properties of the GM-CSF binding sites did not correlate with the FAB classification either, although we tested only 8 AML cases in total. Begley *et al.* studied the binding of recombinant murine granulocyte colony-stimulating factor to AML blast cells and the effect of this factor on clonogenic proliferation[26]; there was no apparent correlation. However, these results should be carefully interpreted, because we observed only the leukemic progenitors in the blast colony assay, and the calculated numbers of the binding sites are the average of the numbers of the whole blast cells, which are apparently heterogeneous, at least in respect to the clonogenicity. If we could study the receptor in homogeneous leukemic progenitors, this matter could be discussed in more detail.

We found that the dissociation constant of the high-affinity binding site was 12–71 p*M*, and that of the low-affinity binding site was 0.5–2.7 n*M* in AML blasts. These results were in good agreement with those obtained from normal monocytes, GM-CSF-responsive leukemic cell lines,[7] and bone marrow mononuclear cells (unpublished data). The reason for the rightward shift of the dose-response curves of blast colony formation was not elucidated in the present study. Several possibilities can be considered to explain the response of AML blasts to higher concentrations of GM-CSF; some cells in the blood from leukemic patients may excrete some substance(s) that lowers the receptor-ligand binding; uncoupling of the receptor-effector system may happen[27, 28]; or the low-affinity receptors may be functional in the AML blasts, at least in the leukemic progenitors. Struc-

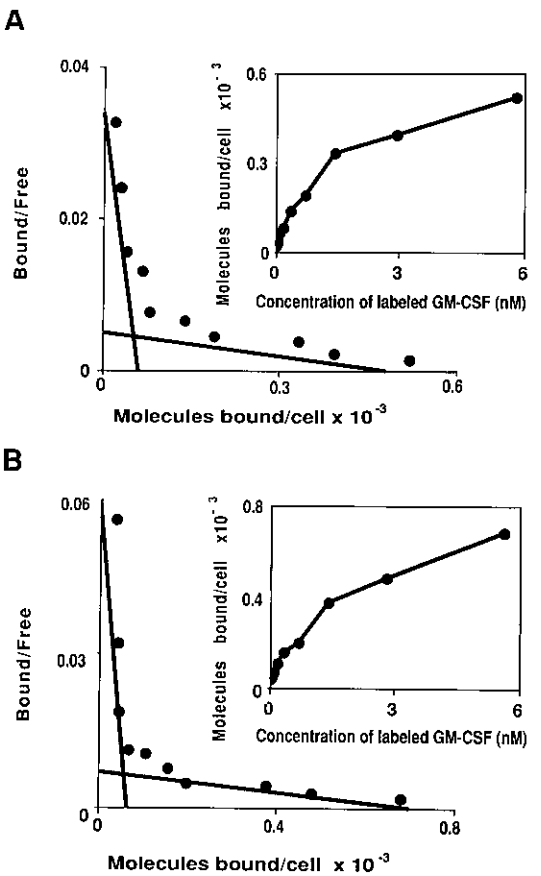

Fig. 4. Scatchard analysis of ^{125}I-GM-CSF-binding to neoplastic lymphoid cells. Cells (2×10^6/200 μl binding buffer) were incubated with various concentrations of ^{125}I-GM-CSF in the presence or absence of an excess of unlabeled GM-CSF. Biphasic Scatchard curves were demonstrated in two cases.

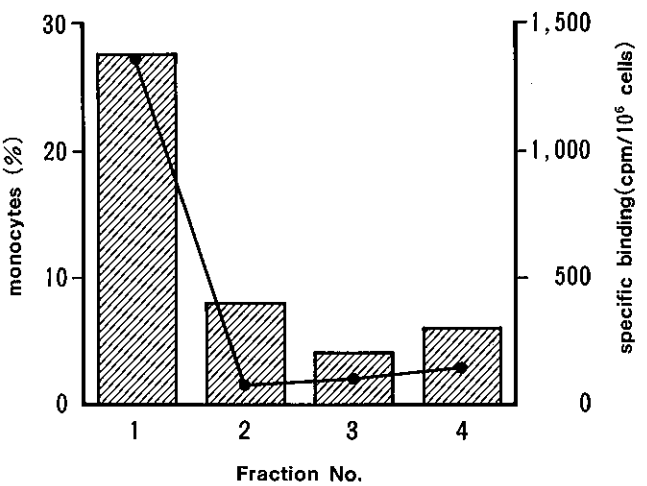

Fig. 5. Binding of ^{125}I-GM-CSF to normal lymphocytes. Cells (2×10^6/200 μl binding buffer) were incubated with ^{125}I-GM-CSF (approximately 3 n*M*) in the presence or absence of an excess of unlabeled GM-CSF. Fraction No. 1, mononuclear; 2, non-adherent; 3, non-adherent and non-phagocytic; and 4, non-adherent, non-phagocytic and non-T cells. Hatched columns represent contaminating monocytes (% of total cells used for binding) and closed circles represent amounts of specific binding.

tural abnormalities in the GM-CSF receptor molecules are also possible. In a cross-linking study on AML blast cells, the observed bands were very similar in size to those demonstrated for peripheral blood neutrophils.[7] However, minor changes in the receptor molecules, which could not be detected by cross-linking experiments, might have occurred.

We confirmed that normal peripheral blood lymphocytes had very little or no specific receptor for GM-CSF. However, two classes of binding site were demonstrated for certain neoplastic lymphoid cells, and the dissociation constants of the binding sites on these cells were very similar to those of the sites on AML blasts. Since monocytes have a significant number of GM-CSF binding sites,[7] we can not neglect the possibility that contamination by a small number of monocytes was responsible for these results. However, in the cases in which ^{125}I-GM-CSF specifically bound to the cells, white blood cell counts were $2\times10^5/\mu$l and $7\times10^4/\mu$l, and more than 95% of these cells were morphologically homogeneous leukemic cells in each case. Therefore, the possibility discussed above seems unlikely. It has been reported that the action of GM-CSF is limited to cells of myeloid and macrophage lineages, and there is no previous report which directly demonstrated that cells of lymphoid lineage respond to GM-CSF in the human system. In the murine system, however, some authors have reported that the proliferation of T cell line HT2 was augmented by murine GM-CSF,[8,9] and Park *et al.* have reported that ^{125}I-labeled murine GM-CSF bound to some murine T cell lines.[10] Thus, similar phenomena could be possible in the human system.

Among the two cases in which we demonstrated the existence of GM-CSF receptor, one expressed immature B-cell phenotype, and the other mature B-cell phenotype (Table I). Normal peripheral lymphocytes did not seem to have the receptor for GM-CSF, as described in the present results. These results suggest that GM-CSF receptor may be expressed as a form of lineage infidelity in some cases of lymphoid malignancies. Further studies will be necessary to understand in more detail the cellular and tissue distributions of GM-CSF receptor, its structure-function correlation, and the physiological and pathognomonic significance of expression of GM-CSF receptor gene.

ACKNOWLEDGMENTS

We thank Dr. Toshio Kitamura for useful discussions, Dr. Hiroaki Honda for performing Southern blot analysis, and Dr. Yoshitomo Muto in Toranomon Hospital for providing clinical materials. This work was supported in part by a Research Grant for Intractable Diseases from the Ministry of Health and Welfare of Japan, and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

(Received May 20, 1989/Accepted July 12, 1989)

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