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Binding of the differentiation-inducer, granulocyte-colony-stimulating factor, to responsive but not unresponsive leukemic cell lines

(iodination/Scatchard analysis/competition/receptor/bone marrow)

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ABSTRACT **Granulocyte-colony-stimulating factor (G-CSF) is a tissue-derived 25,000 M_r glycoprotein that stimulates neutrophilic granulocyte colony formation from murine bone marrow progenitor cells *in vitro*. It is also a potent inducer of terminal differentiation and suppressor of stem cell renewal in the murine myelomonocytic leukemic cell line WEHI-3B. Purified G-CSF was radioiodinated to high specific radioactivity with retention of full biological activity. Iodinated G-CSF bound specifically to WEHI-3B cells, J774 macrophage tumor cells, and normal murine bone marrow cells but not to a variety of other tumor cell lines or murine thymocytes. WEHI-3B cells showed a high affinity for ^{125}I-labeled G-CSF (K_d = 90 pM) but displayed only a small number of specific receptors (300–700 per cell) at 37°C. Other purified colony-stimulating factors showed no competition for binding to these receptors. WEHI-3B (D^-), a subline of WEHI-3B that cannot be induced to differentiate by G-CSF, showed no specific binding of this factor, indicating that it is deficient in receptor presentation.**

Colony-stimulating factors are a family of hemopoietic regulatory growth factors required for the survival, proliferation, and differentiation *in vitro* of hemopoietic progenitor cells (1). The same factors also stimulate metabolic and functional activities of postmitotic mature hemopoietic cells (2–5). Four types of colony-stimulating factor have been purified from murine tissue sources and can be distinguished by unique molecular and biological properties (6). M-CSF stimulates nearly exclusively macrophage colonies to develop from bone marrow precursor cells (7); GM-CSF stimulates neutrophilic granulocyte and macrophage colonies (8); Multi-CSF [also known as burst-promoting activity (9), interleukin 3 (10), P-cell stimulating factor (11), mast cell growth factor (12), and hemopoietic cell growth factor (13)] stimulates neutrophilic and eosinophilic granulocyte, macrophage, erythroid, megakaryocyte, and mast cell colonies; and G-CSF stimulates predominantly neutrophilic granulocyte colonies (14, 15).

Recently, there has been interest in the possibility of using normal hemopoietic growth factors to suppress myeloid leukemias by inducing terminal differentiation in the leukemic cells (16–19). The purification to homogeneity of the factor in mouse-lung-conditioned medium able to induce differentiation in the murine myelomonocytic leukemic cell line WEHI-3B (D^+) (14) indicated that it was separable from GM-CSF and was identical to G-CSF (14). Purified G-CSF induced the production of differentiated cells from, and suppressed the self-renewal capacity of, WEHI-3B (D^+) leukemic stem cells (20) while GM-CSF had weak differentiation-inducing activity and M-CSF and Multi-CSF had none. On the other hand, G-CSF had no detectable effects on a differentiation-unresponsive subline of WEHI-3B (D^-) (20).

To understand better the specificity and action of G-CSF on normal and leukemic cells we have radioiodinated purified G-CSF and measured its binding to normal hemopoietic cells and various leukemic cell lines.

MATERIALS AND METHODS

Cells and Cell Lines. Differentiation-responsive (D^+) and unresponsive (D^-) sublines of the BALB/c murine myelomonocytic leukemia WEHI-3B were maintained as described (20). Other tumor cell lines were kindly supplied by A. W. Harris of this institute and were harvested during the exponential growth phase. All normal murine cells used in this study were obtained from C57BL/6f/J/WEHI mice (3–4 months old, either sex).

Colony-Stimulating Factors. GM-CSF and M-CSF were purified to homogeneity by modifications of reported techniques (8, 21) and were gifts from A. W. Burgess. Multi-CSF purified to homogeneity from pokeweed mitogen-stimulated murine spleen cell conditioned medium and Multi-CSF highly purified (>100,000-fold) from WEHI-3B (D^-) conditioned medium were gifts from R. L. Cutler.

Preparation of Iodinated G-CSF. G-CSF was purified from mouse-lung-conditioned medium as described (14) and consisted of one major silver-stained band migrating with an apparent M_r of 25,000 on NaDodSO$_4$/polyacrylamide gels. Amounts of 1 μg or less were iodinated in 150 μl by using a two-phase method described by Tejedor and Ballesta (22) except that 12 μl of 0.125 mM KI was added with 10 μl of carrier-free Na^{125}I (1 mCi; 1 Ci = 37 GBq) and the filter paper was changed three times over a 40-min incubation period at room temperature. Reactants were separated from labeled G-CSF by passage of the reaction mixture through a small column of Sephadex G-25 medium (Pharmacia, Uppsala, Sweden) equilibrated in sodium phosphate-buffered (0.02 M, pH 7.3) saline (0.15 M) containing Tween 20 (0.02%). Just before use, this material was diluted 1:15 with 10 mM sodium phosphate buffer adjusted to pH 2.6 with citric acid containing Tween 20 (0.02%) and applied to a small column (300 μl) of CM-Sepharose CL-6B (Pharmacia) equilibrated in the same buffer. After elution with 5 ml of phosphate/citrate buffer [to elute residual free iodide, iodotyrosine, and, possibly, iodinated Tween 20 (30–40% of the applied radioactive material)], the bound iodinated G-CSF (^{125}I-G-CSF) was eluted with phosphate-buffered saline containing Tween 20 and used in the binding experiments.

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Abbreviations: G-CSF, granulocyte-colony-stimulating factor; GM-CSF, granulocyte–macrophage-colony-stimulating factor; M-CSF, macrophage-colony-stimulating factor; Multi-CSF, colony-stimulating factor with multiple biological specificities; D^+, differentiation-responsive; D^-, differentiation-unresponsive.

Biological and Molecular Characteristics of ^{125}I-G-CSF. Aliquots of the same batch of purified G-CSF were taken; one aliquot was labeled and processed as above while the other was diluted to the same final concentration in phosphate-buffered saline containing Tween 20. Each preparation was assayed in parallel for its ability to stimulate murine bone marrow colony formation or to induce differentiated colonies of WEHI-3B (D^+) cells as described (14). The specific radioactivity of ^{125}I-G-CSF preparations was determined by self-displacement analysis as described (23) with correction for maximal binding capacity of the preparation. The percentage of total cpm added that was associated with ^{125}I-G-CSF that could specifically bind to WEHI-3B cells (maximal binding capacity) was determined by allowing ^{125}I-G-CSF to bind to WEHI-3B (D^+) cells and sequentially testing the specific binding capacity of the remaining supernatant through two cycles of absorption (24).

Binding of ^{125}I-G-CSF to WEHI-3B and Other Cells. WEHI-3B cells were resuspended in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum (incubation medium) and in these experiments were at least 95% viable as assessed by eosin exclusion. In most cases 1–2 $\times 10^6$ cells in 60–200 μl of incubation medium containing ^{125}I-G-CSF at various concentrations with or without a 20-fold excess of unlabeled G-CSF were incubated for 1.5 hr at 37°C in Falcon 2058 tubes in a fully humidified incubator in 10% CO_2 in air. The tubes were then chilled on ice, and the cells were resuspended and transferred onto 150–200 μl of chilled fetal calf serum in tapered 0.5-ml flexible plastic centrifuge tubes.

The tubes were centrifuged at 340 $\times$ *g* for 5 min and then cut with a scalpel blade 2–3 mm above the cell pellet. The radioactivities of pellet and supernatant were measured separately in a Packard Autogamma 500 C instrument at a counting efficiency of 75% for 1 min or longer if required to reduce the standard deviation to less than 2%. Nonspecific binding, as measured in the presence of (usually) a 20-fold excess of pure unlabeled G-CSF, increased linearly with amount of ^{125}I-G-CSF added and was typically 1–2% of added radioactive factor. For analysis of binding data by the method of Scatchard, the radioactivity associated with unbound (free) ^{125}I-G-CSF was measured directly and corrected for the maximal binding capacity of the preparation.

RESULTS

Radioiodination of G-CSF. Repeated attempts to obtain biologically active preparations of iodinated G-CSF by using chloramine-T or IODO-GEN were unsuccessful. However, as shown in Fig. 1*A*, microgram or submicrogram amounts of pure G-CSF could be iodinated to high specific radioactivity with no detectable loss of colony-stimulating activity for normal cells or differentiation-inducing activity for WEHI-3B (D^+) leukemic cells when the two-phase radioiodination method (22) was used. The figure also shows that, with the inclusion of Tween 20 in all buffers, quantitative recovery of iodinated G-CSF was obtained without the use of protein carrier (14). Iodinated G-CSF could be stored carrier-free at −20°C for at least 1 month with no loss of specific cell-binding capacity.

The total specific binding capacity of ^{125}I-G-CSF (purified through Sephadex G-25 medium and CM-Sepharose CL-6B chromatography) was measured in sequential binding experiments with WEHI-3B (D^+) cells, and the results indicated that 70% of the total radioactivity was associated with molecules able to specifically bind to WEHI-3B (D^+) cells (Fig. 1*B*). Material of this type was used in all subsequent experiments.

The specific radioactivity of ^{125}I-G-CSF preparations was determined by self-displacement analysis of the binding of ^{125}I-G-CSF to WEHI-3B (D^+) cells by increasing concentrations of ^{125}I-G-CSF or unlabeled pure G-CSF. The approximate parallelism of these curves (Fig. 1*C*) indicated that there was no marked difference in binding affinity for WEHI-3B (D^+) cells between ^{125}I-G-CSF and unlabeled G-CSF. The calculated specific radioactivity of ^{125}I-G-CSF preparations was between 240,000 and 450,000 cpm/ng.

Binding Characteristics of ^{125}I-G-CSF to WEHI-3B (D^+) Cells. The amount of ^{125}I-G-CSF specifically bound to WEHI-3B (D^+) leukemic cells or normal murine (C57BL) bone marrow cells was linear with cell number and extrapolated through the origin (Fig. 2*A*).

Specific binding of ^{125}I-G-CSF to WEHI-3B (D^+) cells showed different kinetics and levels of saturable binding at different temperatures (Fig. 2*B*). The binding at 37°C was relatively rapid (complete within 1 hr) and achieved a steady-state level over a 5-hr period. Binding was slower at 23°C,

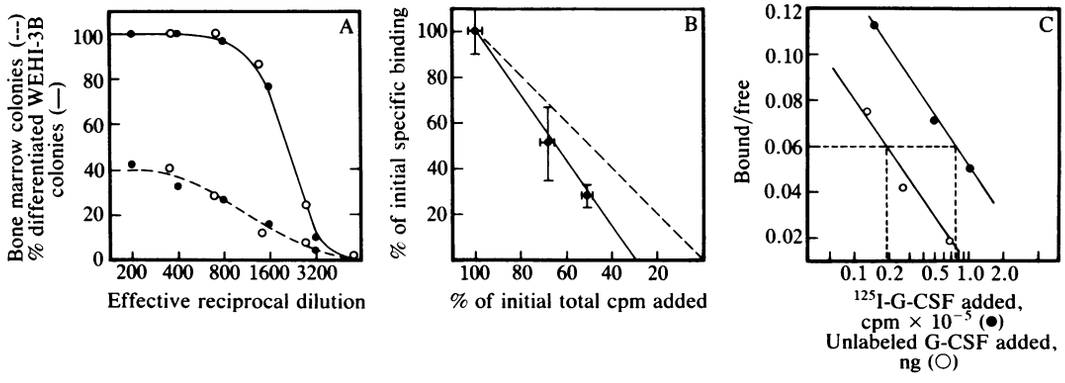

FIG. 1. Characteristics of iodinated G-CSF. (*A*) Ability of iodinated G-CSF (○) (specific radioactivity 4.5 Ci/μmol) and the purified G-CSF used for the radioiodination (●) to induce differentiation in WEHI-3B (D^+) colonies (——) or colony formation by murine bone marrow cells (------). (*B*) Measurement of the maximal binding capacity of ^{125}I-G-CSF. ^{125}I-G-CSF (90,000 cpm) was incubated with 5 $\times 10^6$ WEHI-3B (D^+) cells with or without a 20-fold excess of unlabeled pure G-CSF for 1.5 hr at 37°C. The supernatants of samples without unlabeled G-CSF were assayed for radioactivity and used for rebinding to fresh cells. This procedure was repeated once more and specific binding and the radioactivity added for each binding experiment were expressed as a percentage of the initial values. At least three replicates were used for each point. The experimental intercept on the abscissa is the percentage of total radioactivity incapable of binding to WEHI-3B (D^+) cells (30%). A second experiment gave a value of 20% for radioactivity unable to bind. (*C*) Determination of the specific radioactivity of ^{125}I-G-CSF. ^{125}I-G-CSF (25,000 cpm) and increasing amounts of ^{125}I-G-CSF (up to 120,000 cpm) or unlabeled G-CSF (up to 0.7 ng) were added to 2 $\times 10^6$ WEHI-3B (D^+) cells and incubated for 1.5 hr at 37°C. Specific binding was determined and plotted as a bound/free ratio (corrected for maximal binding capacity). The specific radioactivity was determined as the amount of radioactivity (also corrected for maximal binding capacity) divided by the amount of unlabeled G-CSF added to obtain the same bound/free ratio.

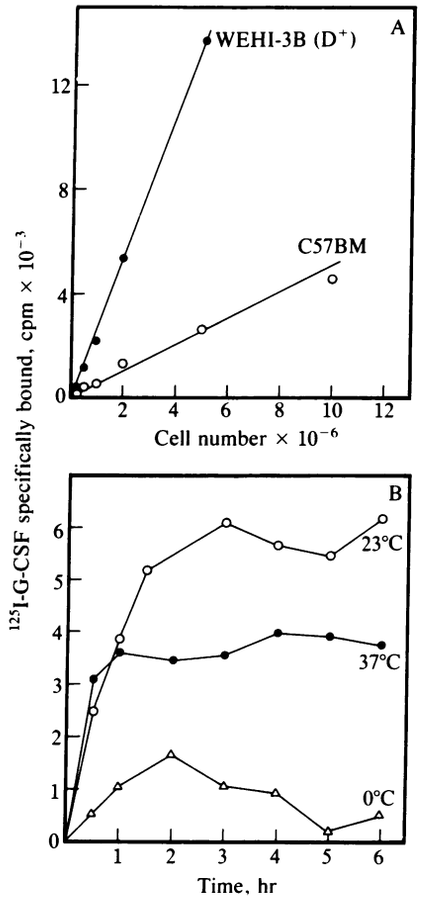

FIG. 2. Effect of cell number, time, and temperature on the binding of ^{125}I-G-CSF to WEHI-3B (D$^+$) cells. (*A*) ^{125}I-G-CSF (150,000 cpm) was incubated with increasing numbers of WEHI-3B (D$^+$) cells (●) or C57BL bone marrow cells (○) in a final volume of 400 μl for 1.5 hr at 37°C. Specific binding (total binding minus binding in the presence of a 20-fold excess of unlabeled G-CSF) was determined. Points are averages for duplicate determinations of both total and nonspecific binding. (*B*) Kinetics of specific binding of ^{125}I-G-CSF to 2 × 10^6 WEHI-3B (D$^+$) cells at 37°C (●), 23°C (○), and 0°C (△). After 3–4 hr of incubation at 0°C extensive cell clumping was observed, so data from these and later time points at 0°C are unreliable. Points are averages of duplicate determinations.

requiring 2 hr to achieve steady-state levels, but the maximal binding level was about 50% higher than at 37°C. The binding at 0°C was relatively slow but reliable steady-state levels could not be obtained because WEHI-3B (D$^+$) cells began to agglutinate after a few hours at 0°C. In other experiments, 1.5 hr was chosen for binding experiments at 37°C to ensure maximal binding but minimize the possibility of receptor turnover (at this time more than 90% of cell-associated ^{125}I-G-CSF was still precipitable with 10% trichloroacetic acid). Continuous shaking of incubation tubes, inclusion of sodium azide (0.02%) to reduce receptor internalization, or inclusion of various protease inhibitors in the incubation medium did not significantly change the level of binding observed over this time period (data not shown).

Binding Specificity of G-CSF. ^{125}I-G-CSF showed no significant specific binding to a variety of tumor cell lines of diverse cellular origin (Table 1). Specific binding was observed with the myelomonocytic leukemic cell line, WEHI-3B (D$^+$), known to respond to G-CSF with differentiation induction but not to the differentiation-unresponsive subline WEHI-3B (D$^-$) (20) (Table 1). ^{125}I-G-CSF also bound specifically to the macrophage tumor cell line J774 but not to two other macrophage and one promonocytic tumor cell lines.

Table 1. Binding of ^{125}I-G-CSF to normal cells and cultured tumor cells

Exp.	Cells or tumor cell line		^{125}I-G-CSF specifically bound per 2 × 10^6 cells: cpm	%
1	WEHI-3B (D$^+$)	(myelomonocytic)	1154	100
	J774	(macrophage)	2029	176
	WEHI-3B (D$^-$)	(myelomonocytic)	−286	−25
	P388 D_1	(macrophage)	−124	−11
	PU-5-1R	(macrophage)	35	3
	WEHI-265.1	(promonocytic)	84	7
	18-81	(pre-B lymphoma)	−57	−5
	WEHI-22.1	(T-lymphoma)	−174	−15
	F4N	(erythroleukemia)	17	1
	P3.6.2.8.1	(plasmacytoma)	31	3
	EMT6	(sarcoma)	113	10
2	WEHI-3B (D$^+$)	(myelomonocytic)	2850	100
	C57BL bone marrow	(normal)	1826	64
	C57BL spleen	(normal)	112	4
	C57BL thymus	(normal)	−99	−3

Cells (2 × 10^6) were incubated in a volume of 70 μl containing ^{125}I-G-CSF (30,000 cpm) with or without unlabeled G-CSF (20-fold excess) for 1.5 hr at 37°C. Specific binding is total binding minus binding in the presence of excess inhibitor. The results are averages of duplicate tubes with or without unlabeled G-CSF.

The binding to J774 was saturable and reversible with an affinity and receptor number similar to that for WEHI-3B (D$^+$) cells (data not shown), and this line also possesses M-CSF-specific receptors (21). This may reflect the common cellular origin of granulocytes and macrophages and the ability of G-CSF to stimulate a subset of such precursor cells (15). ^{125}I-G-CSF also showed specific binding to normal murine bone marrow cells but not to spleen and thymus cells (Table 1). In other experiments, at higher cell concentrations, thymocytes remained negative but spleen cells showed low but significant binding of ^{125}I-G-CSF. Autoradiographic analysis indicated that, for both spleen and bone marrow, binding was restricted predominantly to granulocytic cells.

The ability of unlabeled G-CSF and other functionally related purified colony-stimulating factors to compete with ^{125}I-G-CSF for binding sites on WEHI-3B (D$^+$) cells is shown in Fig. 3. Unlabeled G-CSF was able to compete in a concentration-dependent manner for 80–90% of the radioactivity bound to WEHI-3B (D$^+$) cells. No significant competition was seen with any other colony-stimulating factor over a comparable concentration range. In other experiments, insulin (1 unit/ml), glucagon (10 μM), multiplication-stimulating activity (0.22 μg/ml), somatostatin (10 μM), epidermal growth factor (6 μg/ml), nerve growth factor (12 μg/ml), and fibroblast growth factor (0.22 μg/ml) failed to modify (within ± 10%) the binding of ^{125}I-G-CSF to WEHI-3B (D$^+$) cells under conditions in which unlabeled G-CSF inhibited total binding by 80%.

Binding of ^{125}I-G-CSF to WEHI-3B (D$^+$) and WEHI-3B (D$^-$) Cells. The specific binding of ^{125}I-G-CSF to WEHI-3B (D$^+$) and WEHI-3B (D$^-$) cells as a function of concentration of ^{125}I-G-CSF added is shown in Fig. 4. The binding curve for D$^+$ cells was approximately hyperbolic but no specific binding of ^{125}I-G-CSF was seen for D$^-$ cells over the range where the steepest rise was seen for D$^+$ cells. At higher concentrations of ^{125}I-G-CSF an exponential increase in binding of ^{125}I-G-CSF to D$^-$ cells was observed. Although this binding was competed for by unlabeled G-CSF, autoradiographs indicated that the labeling shown by D$^-$ cell suspensions was

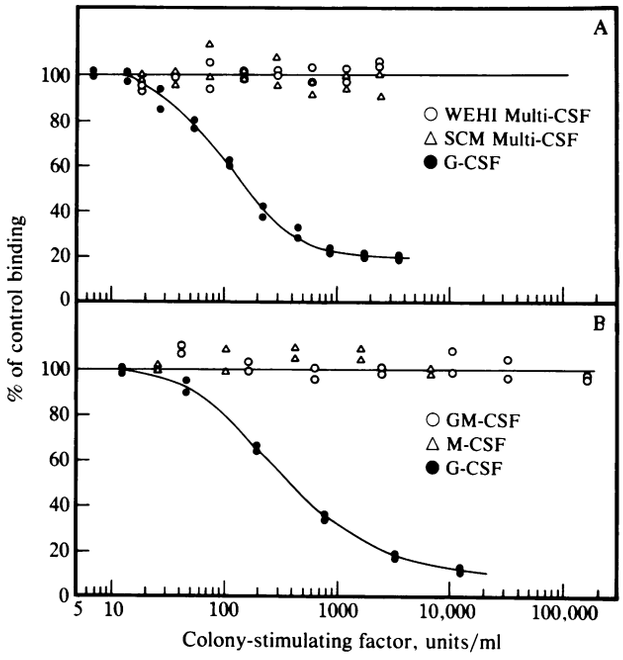

FIG. 3. Inhibition of binding of ^{125}I-G-CSF to WEHI-3B (D^+) cells by pure G-CSF, GM-CSF, M-CSF, and Multi-CSF from WEHI-3B (D^-)-conditioned medium or spleen-conditioned medium (SCM). Binding incubations were for 1.5 hr at 37°C with 2×10^6 WEHI-3B (D^+) cells. Each pure colony-stimulating factor was titrated in bone marrow colony stimulation assays to standardize the specific biological activity of each preparation. The data are plotted as percent of total binding in the absence of any inhibitor and the results of duplicate determinations are shown. In *A* 50,000 cpm of ^{125}I-G-CSF was used and in *B* 110,000 cpm was used.

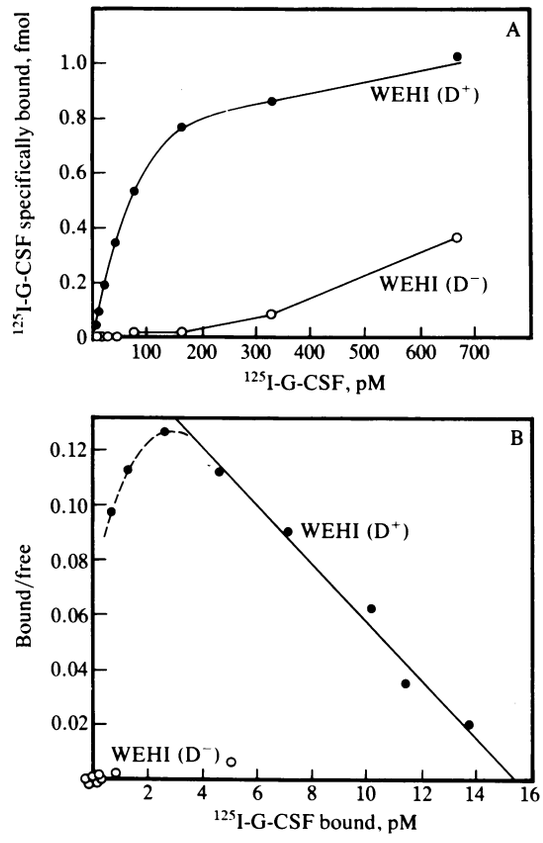

FIG. 4. Binding of ^{125}I-G-CSF to WEHI-3B (D^+) (●) and WEHI-3B (D^-) (○) cells as a function of the concentration of ^{125}I-G-CSF added. Cells (2×10^6 in 76 μl) were incubated at 37°C for 1.5 hr. Specific binding was determined by subtracting the binding in the presence of a 20-fold excess of unlabeled G-CSF. The specific radioactivity of the ^{125}I-G-CSF used was 7×10^{18} cpm/mol. (*A*) Binding curves. (*B*) Scatchard plot of the same data. The average of duplicate determinations is shown. This experiment was repeated twice with similar results.

associated with dead cells and debris, in contrast to the clear labeling of intact D^+ cells (Fig. 5).

Analysis of the binding data by the method of Scatchard (25) showed that the binding of ^{125}I-G-CSF to WEHI-3B (D^+) cells was bimodal, the negative inverse of the linear portion giving a dissociation constant of 94 pM, with the intercept on the abscissa yielding a value of 350 sites per cell (Fig. 4*B*).

In three other experiments the dissociation constant varied between 60 and 110 pM and the average number of binding sites per cell between 300 and 700. Since similar results were obtained in serum-free medium and since under these conditions WEHI-3B (D^+) cells did not secrete detectable levels of G-CSF, this low number of receptors was probably not due to occupation of receptors prior to the experiment. The nonlinearity at low concentrations of bound ^{125}I-G-CSF (Fig. 4B) has also been observed in other binding systems and could reflect positive cooperativity of binding, increased degradation of ^{125}I-G-CSF, or incomplete equilibration of radiotracer with cells at low concentrations (24). Scatchard analysis of binding to WEHI-3B (D^-) cells did not reveal the presence of specific binding sites.

DISCUSSION

^{125}I-G-CSF bound to the responsive leukemic cell line WEHI-3B (D^+) and to normal target cells in murine bone marrow. The specificity of these interactions was indicated by the absence of detectable binding to the differentiation-unresponsive subline WEHI-3B (D^-), to several tumor cell lines of different cellular origin, and to murine spleen and thymus cells.

Moreover, the specific binding of ^{125}I-G-CSF to WEHI-3B (D^+) cells was inhibited by unlabeled G-CSF but not by other functionally related colony-stimulating factors or other growth factors. Interestingly, the highly purified Multi-CSF produced by WEHI-3B (D^-) cells did not inhibit binding by ^{125}I-G-CSF, eliminating the possibility that this factor contributes to the differentiation-unresponsiveness of the subline by blocking the G-CSF receptor.

In contrast to reports of the binding of ^{125}I-M-CSF to peritoneal macrophages or bone marrow cells (21, 26–28), the binding of ^{125}I-G-CSF to WEHI-3B (D^+) cells was rapid, reaching a stable maximum within 1 hr at 37°C. The number

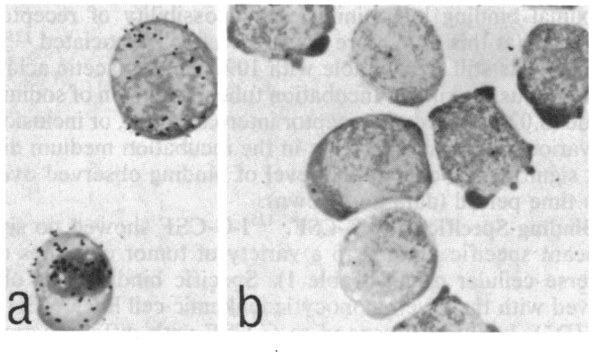

FIG. 5. Autoradiographs of WEHI-3B (D^+) cells (*a*) and WEHI-3B (D^-) cells (*b*) labeled in parallel with ^{125}I-G-CSF (1.5 hr, 37°C) and processed identically (4-week exposure to Kodak NTB2 emulsion). Note the labeling of (D^+) but not (D^-) cells. (×800).

of receptors detected on WEHI-3B (D^+) cells was surprisingly low (300–700 per cell) compared to 70,000 receptors for M-CSF on peritoneal macrophages (21, 27). The binding of ^{125}I-G-CSF to WEHI-3B (D^+) cells was of high affinity (dissociation constant 60–110 pM) and revealed only one major type of binding site. Since a half-maximal response to G-CSF as measured by the induction of differentiation in WEHI-3B (D^+) cells or the stimulation of proliferation of normal progenitor cells occurs at a concentration of only 3 pM (14), it appears that these biological responses can occur at a low receptor occupancy (50% response at 10–35 receptors occupied per cell). However, higher concentrations of G-CSF (and hence higher receptor occupancy) do have additional biological effects such as increasing the number of progeny cells generated and recruitment of additional precursor cells (15).

The lack of cross-reactivity of any of the colony-stimulating factors with the G-CSF receptor on WEHI-3B (D^+) cells was also surprising since each of these factors (with the exception of M-CSF) can also stimulate granulocyte colony formation, and they appear to be functionally related. On the other hand, M-CSF and Multi-CSF have no detectable activity on WEHI-3B (D^+) cells, while GM-CSF has only weak differentiation-inducing activity (15). Moreover, G-CSF, GM-CSF, and Multi-CSF have been shown not to cross-react with the M-CSF receptor on macrophages (26), although both GM-CSF and Multi-CSF can stimulate macrophage colony formation (6). This suggests that certain hemopoietic cells simultaneously express separate receptors for several colony-stimulating factors.

The most striking observation in the present study was the absence of specific binding activity for ^{125}I-G-CSF from the differentiation-unresponsive subline WEHI-3B (D^-). The inability of this cell line to differentiate in response to G-CSF may therefore be specifically caused by alteration or loss of the G-CSF receptor.

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