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Reinforcement Effect of Histamine on the Differentiation of Murine Myeloblasts and Promyelocytes: Externalization of Granulocyte Colony-Stimulating Factor Receptors Induced by Histamine

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SUMMARY

Histamine and recombinant granulocyte colony-stimulating factor (rG-CSF) stimulated the differentiation of murine myeloblasts and promyelocytes to mature neutrophils. In connection with this, myeloperoxidase activity of these progenitor cells was decreased by either histamine or rG-CSF treatment. After pretreatment with histamine at 1 μM, both differentiation and the decrease in myeloperoxidase activity of myeloblasts and promyelocytes induced by rG-CSF were significantly augmented. Binding assays using ^{125}I-labeled rG-CSF showed that the number of rG-CSF binding sites on the surface of neutrophil progenitor cells increased after histamine treatment. The histamine-induced increase in rG-CSF binding appeared to be definitely through H_2 receptors. Furthermore, the increase in rG-CSF binding sites due to histamine treatment seemed to take place in association with the externalization of G-CSF receptors, because 1) the binding increase was observed in the presence of cycloheximide, 2) no concomitant increase in [^{3}H]leucine uptake was elicited, and 3) colchicine and cytochalasin D effectively prevented the increase in rG-CSF binding due to histamine. In neutrophil progenitors, cAMP contents increased very rapidly and significantly after either histamine or rG-CSF treatment. Moreover, dibutyryl-cAMP increased rG-CSF binding to neutrophil progenitor cells in a dose-dependent fashion. However, when progenitor cells were pretreated with protein kinase A inhibitors, the histamine-induced increase in rG-CSF binding was remarkably decreased. This result seems to indicate that the stimulatory effects of histamine on rG-CSF binding to progenitor cells are intimately related to the cAMP-protein kinase A system in neutrophil progenitors. Moreover, c-*myc* mRNA expression in neutrophil progenitors was markedly reduced by either histamine or rG-CSF treatment. It was concluded that rG-CSF-induced differentiation of murine neutrophil progenitors was augmented by histamine pretreatment mainly due to an increase in rG-CSF receptors on these cells and this increase might be related to the externalization of rG-CSF receptors.

Production of neutrophils in normal bone marrow is influenced by many factors, such as a variety of cytokines and the microenvironments surrounding the progenitor cells. Thus far, many biological substances that are capable of stimulating the proliferation and differentiation of neutrophil progenitor cells have been reported (1). G-CSF possesses the ability to form colonies, in semisolid agar, that predominantly consist of granulocyte progenitor cells (2). It is known that G-CSF induces the differentiation and proliferation of neutrophil progenitors and, consequently, it increases the number of more differentiated cells. On the other hand, we reported that, when murine bone marrow cells were cultured with histamine at concentrations higher than 10^{-8} M, histamine markedly stimulated differentiation and proliferation of neutrophil precursor cells through H_2 receptors (3, 4). Indeed, the coexistence of equimolar H_2 blockers such as cimetidine and ranitidine completely suppressed this histamine effect, although no such inhibition was induced by the combination of histamine and H_1 blocker (3). In many biological responses mediated by H_2 receptors, a histamine-sensitive adenylate cyclase was stimulated, and such responses are mimicked by cAMP analogues, indicating that cAMP is the second messenger (5). Because G-CSF and histamine have almost the same effects on neutrophil differentiation, it was assumed that there exists some common mechanism for histamine action and G-CSF action on bone marrow cells. In connection with this, it has been reported that exogenous cAMP stimulates bone marrow cells to form colonies *in vitro* (6, 7). The present study was performed to elucidate the mechanism of histamine reinforcement in G-CSF-induced differentiation of neutrophil precursors.

ABBREVIATIONS: G-CSF, granulocyte colony-stimulating factor; rG-CSF, recombinant granulocyte colony-stimulating factor; PBS, phosphate-buffered saline; FCS, fetal calf serum; TCA, trichloroacetic acid; AMT, 3-amino-1*H*-1,2,4-triazole; DMEM, Dulbecco's modified Eagle's medium; SSC, standard saline citrate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CSF, colony-stimulating factor; db-cAMP, dibutyryl-cAMP.

Materials and Methods

Animals. BALB/c male mice (6–8 weeks of age) were purchased from Charles River Japan.

Preparation of cells. The mice were killed by cervical dislocation, and bone marrow cells were flushed from femurs and tibias into 10 ml of cold PBS (135 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). Thereafter, the cells were exposed to an erythrocyte-lysis buffer containing 155 mM NH_4Cl and 17 mM Tris·HCl, pH 7.2, at 4° for 2 min (3). Cells were washed with PBS by repeating centrifugation and were then suspended in 4 ml of RPMI 1640 medium supplemented with 10% FCS (Whittaker M.A. Bioproducts). The cell suspension was poured into a plastic dish covered with FCS and was placed in a humidified atmosphere of 5% CO_2 in air at 37° for 1 hr. Nonadherent cells were collected and fractionated by means of Percoll discontinuous density gradient centrifugation (ρ = 1.06–1.08) (4). Most of the myeloblasts and promyelocytes were collected from the fractions having specific densities of 1.068–1.070 (4).

Cell culture. In most cases, the cells were cultured in RPMI 1640 medium supplemented with 20% heat-inactivated horse serum (3), unless otherwise stated. In some experiments, the effect of rG-CSF was tested in the same culture medium. However, when the neutrophil precursors were tested with drugs other than rG-CSF, the drugs were dissolved in the same medium and, after the incubation was carried out for various periods of time, the cells were washed with the same culture medium a minimum of two times. The degree of differentiation was assessed by using the following equation: differentiation (%) = 100 × [mature neutrophils/(myeloblasts + promyelocytes)].

Cell staining. Cells treated with drugs were suspended at a concentration of 1 × 10^5 cells/ml in PBS containing 10% FCS. Thereafter, cells were spread on a glass slide by means of a cytocentrifuge (Cytospin, Sakura) and stained with May-Grünwald-Giemsa solution for microscopic observation.

Measurement of intracellular cAMP. Cells treated with the drugs were centrifuged at 1200 rpm for 5 min, and 2 ml of 5% TCA were added to the test tube containing the cell pellet. Cells were disrupted by sonication and subsequently centrifuged. To remove TCA, the supernatant was washed with water-saturated ether; 4 ml of ether were added at each time and mixed vigorously and the tubes were centrifuged at 2000 rpm for 1 min. The upper layer was discarded. The washing process was repeated five times. Samples were dried *in vacuo* at 40° and stored at −20° until immediately before use. The measurement of cAMP was performed using a cAMP radioimmunoassay kit (YAMASA).

Measurement of myeloperoxidase activity. Peroxidase activity was measured according to the method of Chance and Mahely (8), with some modifications. Neutrophil progenitors (1 × 10^5 cells) were disrupted in 1 ml of 0.1% Triton X-100 solution and then 2 ml of the reaction mixture, consisting of 13 mM guaiacol, 0.02% cetyltrimethylammonium bromide, and 0.1 M phosphate buffer, pH 7.0, were added. The reaction was initiated by addition of 50 μl of 20 mM H_2O_2, in the presence or in the absence of 50 μl of 150 mM AMT. Peroxidase activity was expressed as moles of tetraguaiacol formed in 1 min/1 mg of protein. Because eosinophil peroxidase activity was diminished in the presence of AMT, the remaining enzyme activity was accounted for as that derived from myeloperoxidase (9).

Radioiodination of rG-CSF. Radioiodination of rG-CSF was performed by using solid-phase glucose oxidase-lactoperoxidase (10). Five micrograms of rG-CSF were mixed with 50 μl of 0.2 M sodium phosphate buffer, pH 7.2, 10 μl of $Na^{125}I$ (1 mCi), and 10 μl of Enzymobeads (Bio-Rad). The reaction was initiated by the addition of 25 μl of 1% β-D-glucose. After incubation for 20 min at room temperature, 25 μl of 5 mg/ml sodium azide were added to the reaction mixture. The reaction mixture was incubated for another 20 min at room temperature and applied to a PD-10 column (Pharmacia). Labeled rG-CSF was eluted using 0.1 M sodium phosphate buffer, pH 7.0, containing 0.2% bovine serum albumin, to produce separation from unreacted substances. The specific activity of ^{125}I-rG-CSF was 65.8 ± 5.7 μCi/μg of protein (five experiments).

Binding assay with ^{125}I-G-CSF. The binding assay with ^{125}I-labeled rG-CSF was performed according to the method of Walker *et al.* (11). Cells (5 × 10^5) were suspended in 200 μl of RPMI 1640 medium supplemented with 10% FCS and 0.02% sodium azide. Ten microliters of ^{125}I-rG-CSF (20,000 cpm) were added to the medium and incubation was continued at 37° for 30 min, in the presence or in the absence of a 1000-fold excess amount of nonlabeled rG-CSF. The cell suspension was then overlaid on 400 μl of Percoll-PBS (ρ = 1.05) and centrifuged at 2000 rpm for 2 min. The cell pellet was separated by cutting the bottom of the tube and the radioactivity bound to the cells was counted in a γ counter (ARC-1000; Aloka). Specific binding was determined by subtracting the binding in the presence of unlabeled ligand from that determined in the absence of rG-CSF. Histamine was diluted with the culture medium. In some cases, the neutrophil precursors were pretreated with histamine and/or other compounds at 37° for 2 hr before the binding assay.

Procedure for the measurement of ^{125}I-G-CSF binding to α-chymotrypsin-treated cells. Murine myeloblasts and promyelocytes were suspended in Krebs-Ringer-HEPES solution (10^6 cells/ml) and α-chymotrypsin was added to produce a final concentration of 1 μg/ml (12). The cells were incubated at 37° for 15 min and the reaction was terminated with 1/10 volume of FCS. The reaction mixture was passed through a stainless steel mesh (100 mesh) to remove aggregated cells and the cells were collected by centrifugation at 150 × *g* for 10 min at 4°. After a dye exclusion test with Trypan blue, the viability of cells was confirmed to be >95%. The cells were washed and suspended in RPMI 1640 medium supplemented with 10% FCS (10^7 cells/ml). Test compounds were then added and incubation was carried out for various periods of time at 37°, in a 5% CO_2 incubator with humidified air. The reaction was terminated by chilling the culture plate and cells were washed three times with ice-cold RPMI 1640 medium. After that, the ^{125}I-G-CSF binding assay was carried out as described above.

[^{3}H]Leucine uptake into neutrophil precursor cells. Neutrophil progenitor cells were suspended at a concentration of 1 × 10^6 cells/ml in leucine-free DMEM supplemented with 20% horse serum (adjusted to pH 7.4 with 10 mM HEPES). One milliliter of the cell suspension was added to each well of a 24-well plate and, after 30 min of incubation, 6.7 pM (1 μCi/ml) [^{3}H]leucine was added to each well. After 30 min of incubation, the cells were stimulated with 1 μM histamine and incubation was continued for various periods of time. The reaction was terminated by addition of ice-cold DMEM containing 1 mM leucine. After aspiration of the medium, the cells were washed three times with ice-cold DMEM and solubilized by addition of 0.5 N NaOH. The lysate was neutralized with 0.5 N HCl and the radioactivity was determined by means of a liquid scintillation counter (LSC-700; Aloka).

Analysis of c-*myc* mRNA expression. Total RNA extraction from neutrophil progenitor cells was performed by means of guanidium thiocyanate homogenization and lithium chloride precipitation procedures (13). Cells were homogenized in Tris-EDTA buffer containing 4 M guanidium thiocyanate and 20 mM 2-mercaptoethanol. Total RNA was precipitated with ethanol and lithium chloride, successively. Thereafter, phenol-chloroform extractions were carried out. Total RNA precipitated with ethanol was stored in liquid nitrogen until immediately before use. RNA for c-*myc* was detected using v-*myc* biotinylated by means of photoaffinity labeling (14). The v-*myc* (100 μg/ml) was mixed with 1/10 volume of biotin photoprobe (1 mg/ml) and the labeling reaction was developed by irradiation in an ice-bath using a 500-W reflex lamp for 15 min. Nonreacted biotin was removed by repeated 2-butanol extractions. Biotin-bound v-*myc* was collected by ethanol precipitation and stored. Total RNA was diluted to desired concentrations with 2× SSC (1× SSC consisted of 150 mM NaCl and 15 mM trisodium citrate, pH 7.0) and was transferred to a nylon membrane (Genescreen; New England Nuclear) by using a slot blot apparatus (MilliBlot-S; Millipore). The membrane was baked for 2 hr at 80°. The blot was hybridized with biotin-v-*myc* probe for 36 hr at 42°. Thereafter, the

membrane was washed and biotin-v-*myc* on the membrane was detected with horseradish peroxidase-avidin complex. Finally, the membrane was stained for 10–20 min in PBS containing 500 μg/ml 4-chloro-1-naphthol, 500 μg/ml diaminobenzidine, 5% methanol, and 0.003% H_2O_2.

Chemicals. The compounds used were as follows (sources were as indicated): histamine dihydrochloride (Wako Pure Chemicals, Osaka, Japan), pyrilamine maleate (ICN Laboratories, Covina, CA), cimetidine hydrochloride (Fujisawa, Osaka, Japan), ranitidine hydrochloride (Yamanouchi Pharmaceuticals, Tokyo, Japan), KT-5720 and calphostin C (Kyowa Hakko Co., Tokyo, Japan), HA1004 (Seikagaku Kogyo, Tokyo, Japan), cytochalasin D and colchicine (Sigma Chemical Co., St Louis, MO), Percoll (Pharmacia, Uppsala, Sweden), human rG-CSF (YPY-G-CSF) (kindly provided by Kirin Beer Corporation, (Tokyo, Japan), the cAMP assay kit (Yamasa, Tokyo, Japan), a 1.52-kilobase *Pst*I fragment of avian myelocytomatosis virus MC29 proviral DNA (v-*myc*; Takara Biochemicals, Tokyo, Japan), and biotin photoprobe and horseradish peroxidase-avidin (Vector Laboratories, Burlingame, CA).

Statistical analysis. A one-way analysis of variance with Dunnett's test was used to determine statistical significance.

Results

Changes in intracellular cAMP level in myeloblasts and promyelocytes. Fig. 1a shows the changes in intracellular cAMP contents in myeloblasts and promyelocytes treated in the presence or the absence of histamine. In the resting state, the cAMP level in these cells was 780 ± 12 fmol/10^6 cells. However, a significant increase in intracellular cAMP was observed 30 min after exposure to histamine (1 μM). This increase reached the maximal level of 1.22 pmol/10^6 cells 1 hr later. However, no changes in cAMP level were found in the control cells. When rG-CSF (5 ng/ml) was used instead of histamine, a marked elevation of intracellular cAMP levels was also observed. The increase in cAMP reached the maximum (1.28 pmol/10^6 cells) within 30 min and thereafter decreased rather rapidly (Fig. 1b).

Changes in populations of murine myeloblasts and promyelocytes. After neutrophil precursors had been incubated with 1 μM histamine for 2–24 hr, the cells were washed and cultured in fresh culture medium for 24 hr. As shown in Fig. 2a, histamine induced no stimulatory effect on cell differentiation within 2 hr of treatment. However, significant differentiation was observed when the cells were treated with histamine for longer than 4 hr. In another experiment, cells were similarly pretreated with histamine (1 μM) for 2 hr and then progenitor cells were washed and incubated for 24 hr in the same medium containing various concentrations of rG-CSF. As shown in Fig. 2b, the differentiating effect of rG-CSF was observed at a concentration of 1 ng/ml and the differentiation increased dose-dependently. On the other hand, in the histamine-pretreated group rG-CSF caused significant differentiation at a concentration of 0.5 ng/ml. As the concentration of rG-CSF increased, the extent of differentiation in the histamine-pretreated group became more evident than in the group treated with rG-CSF alone. The maximal effect was achieved at 5 ng/ml in the histamine-pretreated group, whereas in the group treated with rG-CSF alone maximal differentiation was achieved at 10 ng/ml. The results seem to indicate that histamine increases the susceptibility of progenitor cells to rG-CSF.

Changes in myeloperoxidase activity of neutrophil precursors. Fig. 3a shows the changes in the myeloperoxidase activity of murine myeloblasts and promyelocytes treated in the presence or the absence of histamine, and Fig. 3b shows the changes in cells incubated with various concentrations of rG-CSF for 24 hr with or without histamine pretreatment for 2 hr. Usually, the myeloperoxidase activity of neutrophil precursors reaches a maximal level at the stage of promyelocytes, and as the differentiation proceeds further the enzyme activity decreases.

As shown in Fig. 3a, myeloperoxidase activity was not altered by 2 hr of histamine treatment. However, a significant decrease was observed after 4 hr of incubation, and the enzyme activity decreased to nearly half that of the control after 24 hr of incubation. In rG-CSF-treated cells, myeloperoxidase activity decreased dose-dependently and rG-CSF treatment at a concentration of 5 ng/ml decreased the enzyme activity to less than half of that of the control. As in the case of morphological changes, the reduction of enzyme activity was more remarkable in the histamine-treated group than in the group treated with rG-CSF alone, and the enzyme activity decreased to the mini-

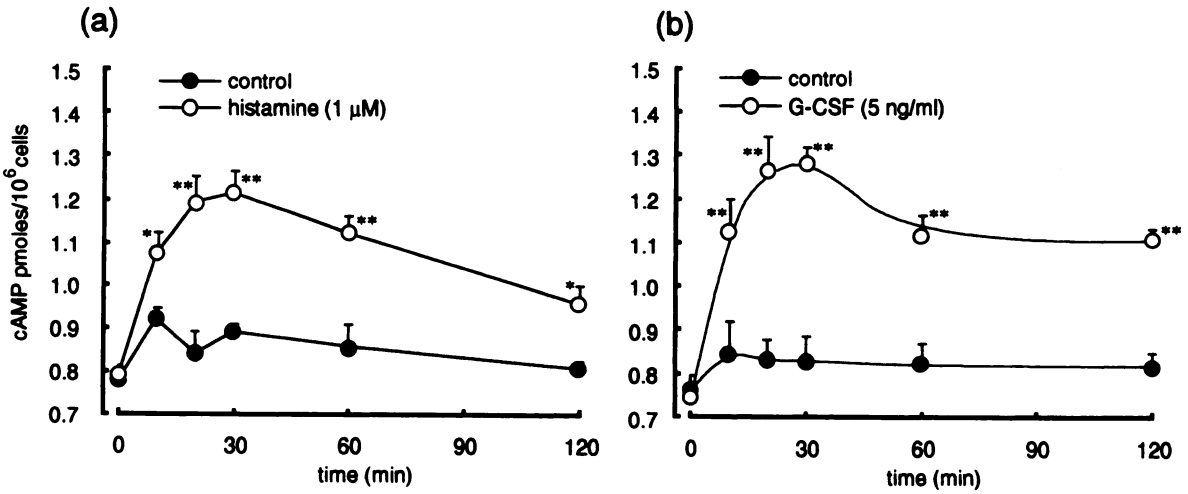

Fig. 1. Effects of rG-CSF and histamine on intracellular cAMP contents in murine myeloblasts and promyelocytes. Cells were treated with histamine (1 μM) (a) or rG-CSF (5 ng/ml) (b) at 37°. The reaction was terminated by the addition of 5% TCA and, thereafter, cAMP contents were determined by using a radioimmunoassay kit. ○, Cells treated with either histamine (a) or rG-CSF (b); ●, nontreated control. Each value is the mean ± standard error from five separate experiments. *, $p < 0.05$; **, $p < 0.01$.

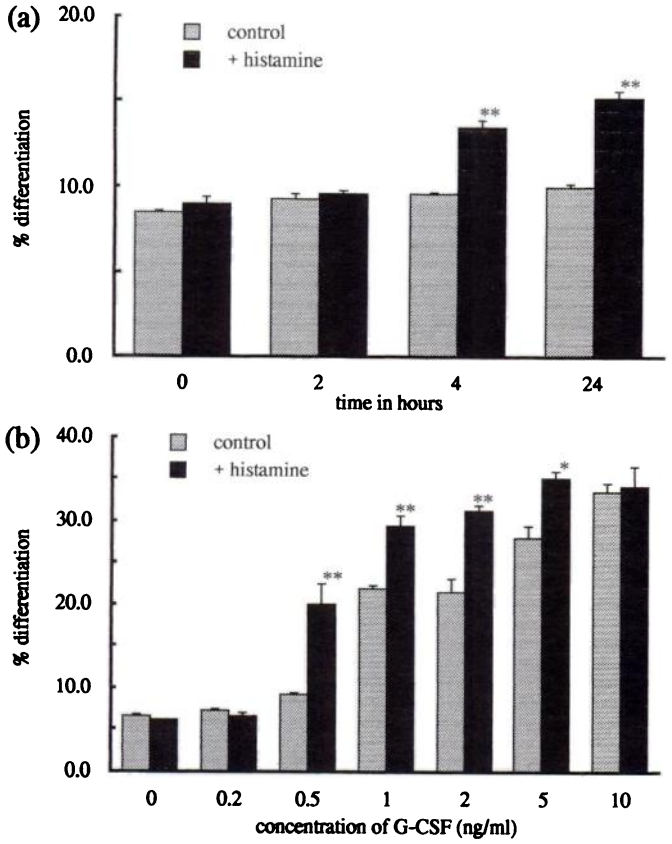

Fig. 2. Changes in the population of mature neutrophils. Murine myeloblasts and promyelocytes were incubated with histamine (a) or rG-CSF (b), with or without histamine (1 μM) pretreatment. The percentage of neutrophils was used as an indicator of cell differentiation. In a, the control value was determined in medium without histamine, and histamine incubation was continued for various periods of time. In b, when the cells were cultured in medium containing rG-CSF alone, the percentage of neutrophils was taken as the control. and**, Significant difference between the control and histamine-pretreated groups, with $p < 0.05$ and $p < 0.01$, respectively. Each value represents the mean ± standard error from five separate experiments.

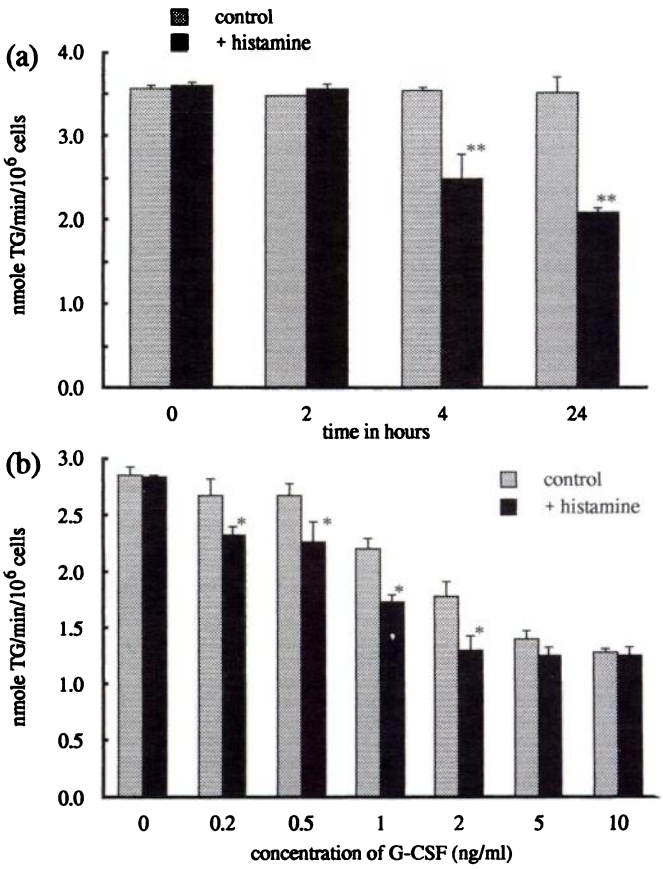

Fig. 3. Changes in myeloperoxidase activity in murine myeloblasts and promyelocytes. a, Sequential changes in the enzyme activity of the medium in both the presence and the absence of histamine (1 μM). The difference between the two groups became significant when incubation was continued for longer than 4 hr. **, $p < 0.01$. b, Comparison of the reduction in enzyme activity between the control (only rG-CSF-treated) group and the histamine (plus rG-CSF)-pretreated group. *, Significant difference between the two groups, $p < 0.05$. Each value represents the mean ± standard error from five separate experiments. *TG*, tetraguaiacol.

mum at a concentration of 2 ng/ml rG-CSF. The results also suggest that histamine pretreatment augments the action of rG-CSF.

rG-CSF binding to murine neutrophil progenitors. To clarify why the reactivity of progenitor cells to rG-CSF increased after histamine pretreatment, the capacity for rG-CSF binding to the progenitor cells was determined using ^{125}I-labeled rG-CSF. As shown in Fig. 4a, when the time course of ^{125}I-rG-CSF binding to myeloblasts and promyelocytes was determined, ^{125}I-rG-CSF binding reached the maximum at 10 min and then decreased to a stable level at 30 min. Thus, in the following experiment, 30 min was taken as a standard time in the binding assay. When saturable binding of ^{125}I-rG-CSF was measured and Scatchard analysis was then carried out, the binding constant (K_d) and the maximal binding capacity (B_{max}) were 60.6 ± 2.3 pM and 521.7 ± 30 binding sites/cell, respectively (Fig. 4, b and c). When the progenitor cells were treated with histamine (1 μM) for various periods of time and rG-CSF binding was determined, the binding reached the maximum at 30 min of histamine treatment and then declined to the stationary level (Fig. 5a). When Scatchard analysis was performed after 30 min of histamine pretreatment, the K_d and B_{max} of ^{125}I-rG-CSF binding were 63.2 ± 2.04 pM and 1754.8 ± 119.2 binding sites/cell, respectively (Fig. 5, b and c). Although the K_d values were almost the same before and after histamine treatment, an approximately 3.4-fold increase in B_{max} was observed after histamine treatment.

Effects of antihistamines on the histamine-induced increase in rG-CSF binding. Table 1 indicates a dose-dependent increase of rG-CSF binding to neutrophil precursor cells after histamine treatment and its inhibition by pretreatment with histamine antagonists. The rG-CSF binding to immature cells treated with histamine significantly increased at concentrations higher than 1×10^{-7} M, compared with the nontreated control. Pretreatment with cimetidine at 1×10^{-6} M was effective in preventing the histamine (1 μM)-induced increase nearly to control levels. Also, ranitidine (at 10 μM) induced almost complete inhibition of the binding. However, no inhibition was effected by pyrilamine pretreatment, even at 10 μM.

Effects of db-cAMP on rG-CSF binding. To investigate whether such an increase in rG-CSF binding sites on neutrophil progenitor cells takes place in association with the changes in intracellular cAMP contents, the effect of db-cAMP on the specific binding of ^{125}I-G-CSF to the neutrophil precursors was studied. When db-cAMP was added at concentrations of 10–

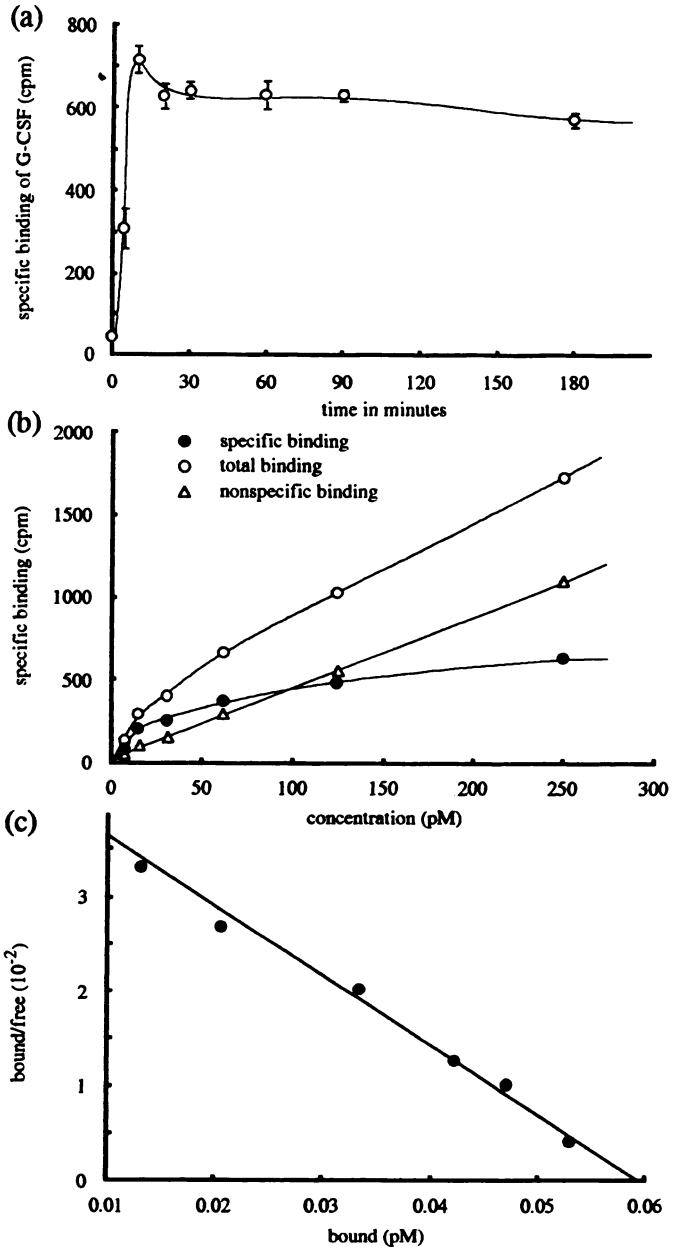

Fig. 4. Specific binding of ^{125}I-rG-CSF to murine neutrophil progenitors. a, Time course of the specific binding of ^{125}I-rG-CSF to myeloblasts and promyelocytes. Each *point* indicates the mean ± standard error of five separate experiments. b, Saturable binding of ^{125}I-rG-CSF to murine myeloblasts and promyelocytes. Each *point* represents the mean of five separate experiments. c, Scatchard analysis.

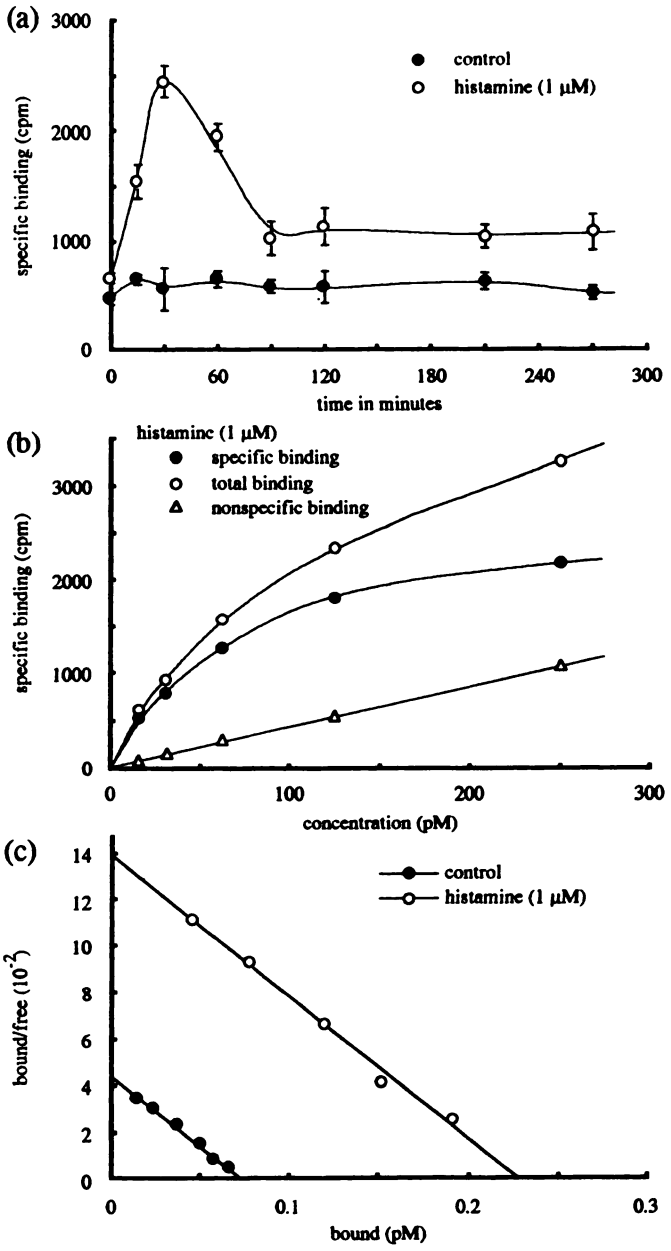

Fig. 5. Specific binding of ^{125}I-rG-CSF to murine neutrophil progenitors treated with histamine (1 μM). a, Sequential changes in specific binding of ^{125}I-rG-CSF to myeloblasts and promyelocytes treated with histamine for various periods of time. Each *point* indicates the mean ± standard error of five separate experiments. b, Saturable binding of ^{125}I-rG-CSF to myeloblasts and promyelocytes treated with histamine for 30 min. Each *point* represents the mean of five separate experiments. c, Scatchard analysis. The two lines are almost parallel to each other. B_{max} for the histamine-treated cells is approximately 3.4 times larger than that for the control cells.

100 μM for 30 min, the specific binding of rG-CSF increased dose-dependently (Fig. 6). The effect of db-cAMP at 100 μM was slightly higher than that detected for histamine at 1 μM.

Effects of protein kinase inhibitors on rG-CSF binding. In many biological reactions, cAMP acts in association with protein kinase A to complete its function. To confirm whether this is the case, the effect of protein kinase inhibitors was tested. When progenitor cells were pretreated for 30 min with KT-5720 (15) or HA-1004 (16), both potent inhibitors of protein kinase A, and were treated with histamine in the following 30 min, rG-CSF binding to neutrophil progenitors was inhibited dose dependently and significantly (Fig. 7, a and b). On the other hand, when calphostin C, a specific inhibitor of protein kinase C (15), was added similarly, in the absence or the presence of histamine, no change in rG-CSF binding was observed (Fig. 7c). This seems to indicate that the increase of rG-CSF binding to neutrophil precursors induced by histamine is intimately related in some way to the cAMP-protein kinase A system.

Effects of protein synthesis inhibitors on rG-CSF binding. It was thought that, when a histamine-induced increase in rG-CSF binding to neutrophil precursors takes place, protein synthesis may occur concurrently. To determine

TABLE 1

Effects of histamine antagonists on the histamine-induced increase in the specific binding of ^{125}I-G-CSF to murine myeloblasts and promyelocytes

After antihistamine pretreatment for 30 min, histamine (1 μM) was added to the medium and incubation was continued for another 30 min (five experiments).

Drugs	Concentration	Specific binding
	μM	cpm
Control	0	550 ± 13
Histamine	0.1	1510 ± 38
	1.0	2347 ± 65
	10.0	2983 ± 245
Cimetidine	0.01	1928 ± 65
	0.1	1477 ± 123
	1.0	619 ± 71
	10.0	534 ± 48
Ranitidine	1.0	1074 ± 26
	10.0	551 ± 103
Pyrilamine	0.01	2659 ± 242
	0.1	2606 ± 39
	1.0	2503 ± 104
	10.0	2370 ± 91

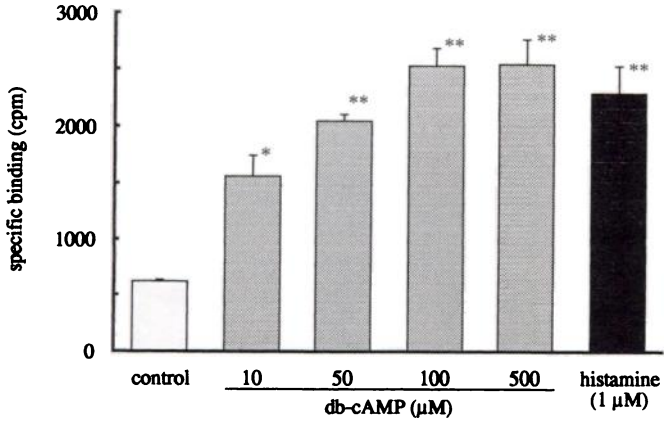

Fig. 6. Effect of db-cAMP on ^{125}I-rG-CSF binding to murine myeloblasts and promyelocytes. After murine myeloblasts and promyelocytes had been incubated with various concentrations of db-cAMP for 30 min, ^{125}I-rG-CSF binding assays were carried out. Each value represents the mean ± standard error of five separate experiments. * and **, Significant difference between the control and db-cAMP-treated groups, with $p < 0.05$ and $p < 0.01$, respectively.

whether this is the case, the cells were pretreated with Actinomycin D or cycloheximide for 30 min, incubation was continued for another 30 min with histamine (10^{-6} M), and rG-CSF binding was carried out similarly. As shown in Table 2, the increase in rG-CSF binding to neutrophil precursors caused by histamine was not inhibited in the presence of either protein synthesis inhibitor. Furthermore, [^{3}H]leucine uptake into myeloblasts and promyelocytes was studied in the presence and in the absence of histamine (1 μM). Histamine had no effect on [^{3}H]leucine uptake. The values determined at 120 min were almost the same for the histamine-treated group and the corresponding control (at 120 min, [^{3}H]leucine uptake in the histamine-treated cells was 9961 ± 456 cpm/10^6 cells, whereas in the control cells it was 10747 ± 425 cpm/10^6 cells; five experiments). The results clearly indicate that rG-CSF binding to progenitor cells takes place without concurrent [^{3}H]leucine uptake. This seems to coincide with the finding that neither Actinomycin D nor cycloheximide inhibited histamine-induced increases in rG-CSF binding.

When neutrophil progenitors were exposed to α-chymotrypsin and incubated with db-cAMP in the presence of cycloheximide (1 μM), ^{125}I-rG-CSF binding increased remarkably, in comparison with the control cells (treated with cycloheximide alone) (Table 3). However, when protease-treated cells were exposed to histamine (1 μM) in the presence of cycloheximide, no such increase in rG-CSF binding was observed (data not shown), suggesting that H_2 receptors either were digested or were inactivated.

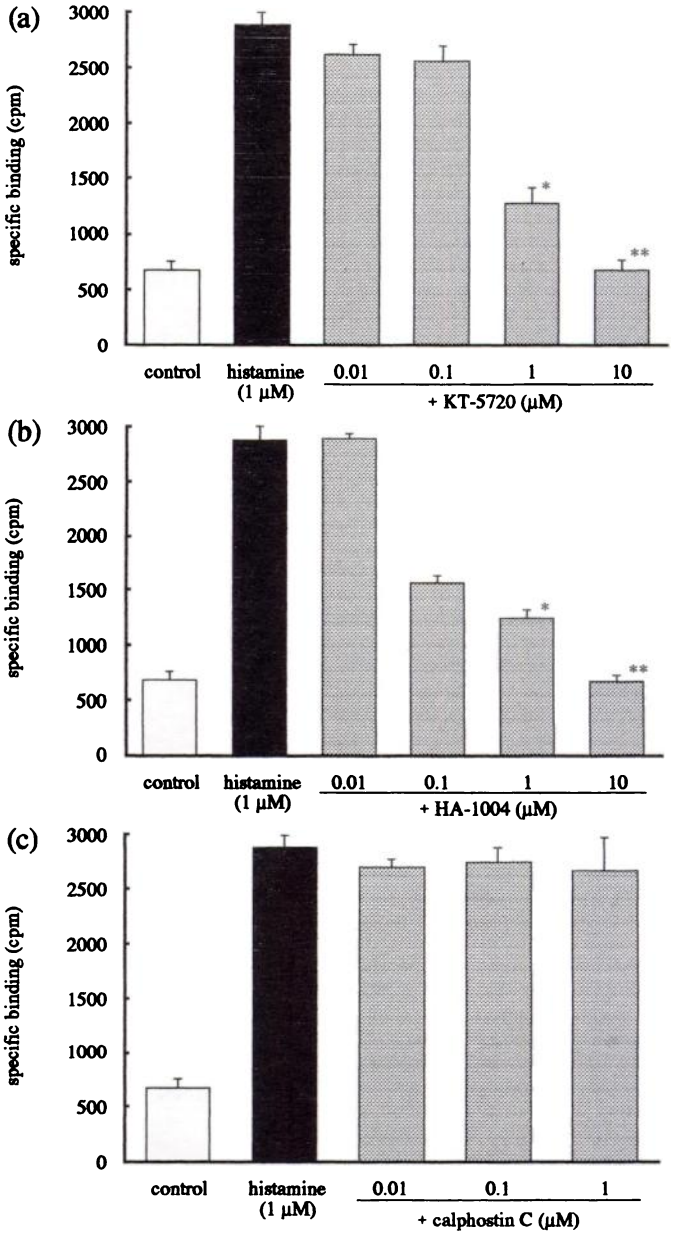

Fig. 7. Effects of protein kinase inhibitors on the histamine-induced increase in ^{125}I-rG-CSF binding to murine myeloblasts and promyelocytes. Effects of KT-5720 (a), HA-1004 (b), and calphostin C (c) are shown. These cells were incubated for 30 min with the inhibitors before histamine addition (1 μM). Each value represents the mean ± standard error of five experiments. * and **, Significant difference between histamine-treated cells and protein kinase inhibitor- plus histamine-treated cells, with $p < 0.05$ and $p < 0.01$, respectively.

Effects of cytochalasin D and colchicine on rG-CSF binding. Because no relation was observed between the increase in rG-CSF receptors and protein synthesis, translocation

TABLE 2

Effects of Actinomycin D and cycloheximide on the histamine-induced increase in the specific binding of ^{125}I-G-CSF to murine myeloblasts and promyelocytes

The cells were pretreated with Actinomycin D or cycloheximide for 30 min and then incubation was continued for another 30 min with histamine (1 μM) (five experiments).

Drugs	Concentration	Specific binding
	μM	cpm
Control	0	756 ± 21
Histamine	1.0	2685 ± 30
Actinomycin D	0.01	2323 ± 376
	0.001	2778 ± 356
	0.0001	2787 ± 171
Cycloheximide	1.0	2672 ± 305
	0.1	2488 ± 156
	0.01	2597 ± 72
	0.001	2571 ± 32

TABLE 3

Effect of db-cAMP on rG-CSF binding to neutrophil progenitors after treatment with α-chymotrypsin

Murine myeloblasts and promyelocytes (10^6 cells/ml) were treated with 1 μg/ml α-chymotrypsin at 37° for 15 min and the reaction was terminated with 1/10 volume of FCS. Thereafter, test compounds were added and incubation was carried out for various periods of time at 37°. After that, the ^{125}I-G-CSF binding assay was performed. Each value represents the mean ± standard error of five separate experiments.

Time	Specific binding of ^{125}I-G-CSF	
	Cycloheximide (1 μM) alone	Cycloheximide + db-cAMP (100 μM)
min	cpm	
0	134 ± 20	132 ± 12
30	135 ± 17	134 ± 24
60	148 ± 15	163 ± 36
120	165 ± 28	296 ± 18*
240	239 ± 23	358 ± 10*
360	277 ± 19	429 ± 15*

* Significant difference, $p < 0.01$.

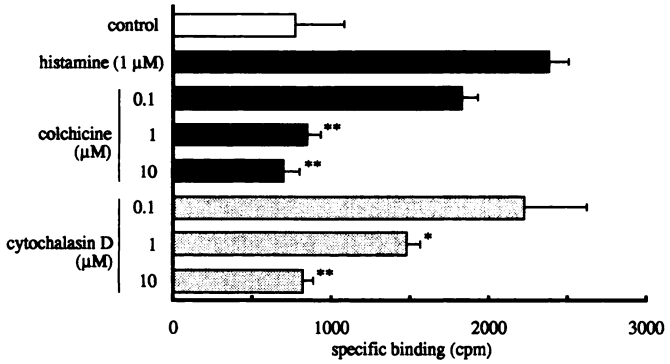

Fig. 8. Effects of colchicine and cytochalasin D on the histamine-induced increase in ^{125}I-rG-CSF binding to murine myeloblast and promyelocytes. The cells were incubated with test drugs for 30 min before histamine addition (1 μM). * and **, Significant difference between histamine-treated cells and test compound- plus histamine-treated cells, with $p < 0.05$ and $p < 0.01$, respectively.

of the receptor from the cell interior to the cell surface (externalization) (12) was considered to be a possible event leading to the receptor increase. To study this possibility, agents acting on the functions of cytoskeletal elements were tested. As shown in Fig. 8, pretreatment with cytochalasin D or colchicine dose-dependently inhibited histamine-induced increases in rG-CSF binding.

Expression of c-*myc* mRNA. It is known that the c-*myc* proto-oncogene is expressed in various immature cells. As shown in Fig. 9a, neutrophil precursor cells expressed the c-*myc* gene. In contrast, mature cells such as neutrophils did not express the oncogene at all. When the neutrophil precursor cells were treated for 0–4 days with rG-CSF at a concentration of 5 ng/ml, c-*myc* gene expression in these cells was augmented after 24-hr treatment. However, gene expression gradually decreased thereafter. Also, when progenitor cells were treated with histamine (1 μM), gene expression markedly diminished within a few days (Fig. 9b).

Discussion

From the present experiments, it became apparent that histamine augments the rG-CSF-induced differentiation of neutrophil precursors mainly through an increase in the number of G-CSF receptors located on the surface of the immature cells. It is known that, when G-CSF binds to a receptor, the ligand-receptor complex is transferred into the cell interior by the internalization process (17, 18). As a consequence of this, down-regulation of the receptors takes place (11). Furthermore, many CSFs are required not only for cell proliferation but also for cell survival (19–21), so that the biosynthesis of receptors may take place actively in these cells. However, in the present experiment, contradictory results were obtained, that is, histamine increased the number of G-CSF receptors on neutrophil progenitors without concomitant protein synthesis (Table 2). The increase in rG-CSF receptors may result from the inhibition of internalization of the receptors or the externalization of receptors (12) from the inside to the outside of the cell surface. In association with this, Deutsch *et al.* (12) reported that, after proteolytic inactivation of cell surface insulin receptors of 3T3-L1 adipocytes, the cells recovered 20% of their external insulin-binding activity within 2 hr, even when protein synthesis was inhibited by cycloheximide. Those authors explained that the pre-existing pool supplies the active insulin

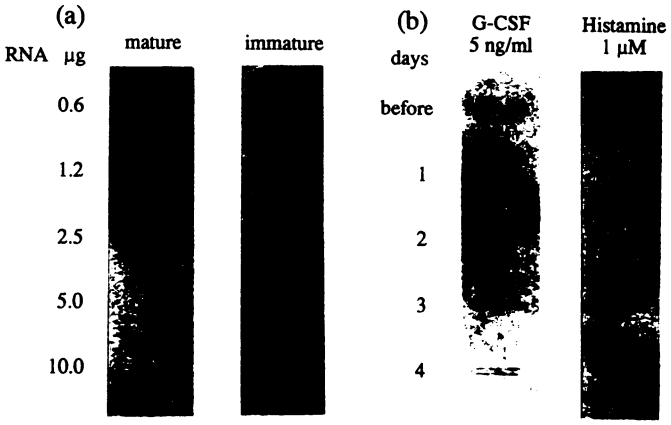

Fig. 9. Effects of rG-CSF and histamine on c-*myc* gene expression in murine myeloblasts and promyelocytes. a, Detection of mRNA for the c-*myc* gene in mature neutrophils and their precursor cells (myeloblasts and promyelocytes). Mature neutrophils were obtained from the peripheral blood of mice. b, Effects of rG-CSF and histamine on c-*myc* gene expression in myeloblasts and promyelocytes. Immature cells were treated with either rG-CSF (5 ng/ml) or histamine (1 μM) for 4 days. Total RNA was isolated and c-*myc* gene expression was determined by slot blot analysis using a biotin-labeled v-*myc* probe.

receptors to the cell surface. When neutrophil progenitors were treated with α-chymotrypsin in the present experiments, ^{125}I-rG-CSF binding in the presence of cycloheximide was markedly decreased, to approximately 20% of the nontreated control. However, addition of db-cAMP significantly increased the binding even in the presence of cycloheximide. These findings seem to suggest that the histamine-induced increase in G-CSF receptors of neutrophil progenitors may be due to externalization of the receptors. Although the exact mechanism is not known, the increase in the number of G-CSF receptors is definitely related to the cAMP-protein kinase A system, as shown in Figs. 6 and 7. Also, as shown in Fig. 8, a histamine-induced increase in G-CSF receptors was markedly inhibited by both cytochalasin D and colchicine. This seems to suggest that either microtubules, microfilaments, or both play an active role in receptor mobilization. Furthermore, a histamine-induced increase in rG-CSF receptors was inhibited by H_2 blockers but not by H_1 blockers (Table 1). This clearly indicates that a histamine-induced increase in rG-CSF receptors takes place in association with the stimulation of H_2 receptors, which may lead to the process increasing intracellular cAMP content. When neutrophil progenitors were treated with α-chymotrypsin, histamine treatment did not increase the rG-CSF binding (data not shown). In this case, H_2 receptors may disappear from the cell surface of neutrophil progenitors and, consequently, no increase in cAMP level is effected.

In the present experiments, human rG-CSF was used instead of murine rG-CSF. It is known that homology between human G-CSF and murine G-CSF is higher than 70% and the biological activities of the two substances are very similar (2). Indeed, as shown in Fig. 2b, rG-CSF caused the differentiation of murine myeloblasts and promyelocytes at concentrations higher than 1 ng/ml. However, when the cells were treated with histamine they became more sensitive to rG-CSF, and cell differentiation occurred even at 0.5 ng/ml.

In the present experiments, a rapid and significant elevation of cAMP was found in murine myeloblasts and promyelocytes treated with either histamine or rG-CSF, as shown in Fig. 1. Furthermore, db-cAMP increased rG-CSF binding to neutrophil precursors in a dose-dependent fashion (Fig. 6). Together with these findings, it was assumed that cAMP plays an essential role in the histamine-induced increase in rG-CSF binding. Indeed, it was reported that db-cAMP is effective not only in increasing the intracellular cAMP level but also in stimulating the differentiation of murine myeloblasts, murine promyelocytes, and human promyelocytic leukemia (HL-60) cells (22). It has been shown that KT-5720 is a specific and potent inhibitor of protein kinase A (15). When KT-5720 was preincubated simultaneously with histamine, the histamine-induced increase in rG-CSF binding was completely inhibited. However, calphostin C, a specific inhibitor of protein kinase C (15), showed no inhibitory effect on the histamine-induced increase in G-CSF receptors. The findings clearly indicate that the increase in rG-CSF receptors may be intimately associated with the cAMP-protein kinase A system.

The c-*myc* gene is a proto-oncogene that is expressed in some tumor cells (23). The product synthesized from this gene is a transcription initiation factor as well as a factor controlling translation (24). There is a report that c-*myc* expression occurred most remarkably in promyelocytes, among the neutrophil lineage cells in humans (25). This may mean that proliferation of neutrophil progenitors takes place most actively at the stage of promyelocytes. In the present study, both rG-CSF and histamine reduced c-*myc* expression in myeloblasts and promyelocytes. It is known that c-*myc* expression is augmented by the activation of protein kinase C (26). As shown in Fig. 1, both histamine and rG-CSF increased the cAMP contents of neutrophil precursor cells. In fact, McCachren *et al.* (27) found that db-cAMP reduces the expression of c-*myc* during HL-60 cell differentiation.

In conclusion, it was considered that histamine increases rG-CSF-induced differentiation of murine neutrophil progenitors mainly through an increase in the binding sites for rG-CSF on these cells, in association with receptor externalization. However, the decrease in c-*myc* expression caused by histamine treatment may well provide another explanation. It seems quite likely that cAMP plays crucial roles in these two events.

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