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Receptor Binding of Human Granulocyte Colony-stimulating Factor to the Blast Cells of Myeloid Leukemia[1]

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ABSTRACT

Human granulocyte colony-stimulating factor (G-CSF) rapidly loses the biological activity and the receptor binding capacity following radioiodination. We have made a mutein of human G-CSF, KW-2228, in which Thr-1, Leu-3, Gly-4, Pro-5, and Cys-17 were respectively substituted with Ala, Thr, Tyr, Arg, and Ser; showed more potent G-CSF activity; and retained full biological activity and receptor binding capacity at least 2 weeks of radioiodination. G-CSF is an effective growth factor for the blasts of myeloid leukemia. Radioiodinated KW-2228 was prepared using solid-phase glucose oxidase-lactoperoxidase. Human leukemia cell lines and the blast cells from leukemia patients were examined for binding. High affinity binding sites were identified on myeloid cell lines and on the blasts obtained from acute myeloid leukemia patients. Scatchard analysis showed that a single binding site for G-CSF was observed (361–1688 receptors/cell; K_d 128–1400 pM). In contrast, specific binding of ^{125}I-KW-2228 was not demonstrated on lymphoblastic cell lines or the blast cells of acute lymphoid leukemia or lymphoma. This difference was reflected in the effectiveness of G-CSF to stimulate colony formation in acute myeloid leukemia blasts, while G-CSF did not stimulate colony formation of the blast cells from acute lymphoid leukemia.

INTRODUCTION

The generation of granulocytes from immature hematopoietic progenitor cells depends on the presence of several hormone-like glycoproteins, the colony-stimulating factors (1). G-CSF[3] is found to be a lineage-specific hematopoietic factor which acts on granulocyte-committed progenitor cells (2). Although G-CSF has been shown to stimulate the growth of nonhematopoietic cells, such as endothelial cells (3), *in vivo* studies support the role of G-CSF as a physiological granulopoietin (2, 4). In human leukemia cells, G-CSF has been shown to stimulate proliferation of leukemic blast cells but it does not induce their differentiation apparently. The inability of G-CSF to induce differentiation of leukemic blast cells is considered to be attributable to abnormalities in G-CSF receptors or postreceptor pathways in the blast cells. To clarify the mechanism of G-CSF action on leukemic cells, we examine the specific binding of human G-CSF to several human leukemia cell lines and fresh leukemic blast cells directly obtained from patients. Since radioiodination of human G-CSF is difficult, we used a molecularly modified G-CSF (5) which retained full biological activity for a long period when it was radioiodinated.

MATERIALS AND METHODS

Preparation of Muteins of Human G-CSF. Clones of complementary DNA encoding human G-CSF were isolated from human cancer cells (6, 7) and from human circulating monocytes as described previously (8). Large quantities of biosynthetic human G-CSF are now available (2, 4). However, intact human G-CSF rapidly loses the biological activity and the receptor binding capacity following radioiodination. In order to obtain muteins of human G-CSF with more stable and potent biological activity, we have made about 100 muteins by site-directed mutagenesis, cassette mutagenesis, insertions, or deletions (5). Among these muteins, KW-2228, in which Thr-1, Leu-3, Gly-4, Pro-5, and Cys-17 (6–8) were respectively substituted with Ala, Thr, Tyr, Arg, and Ser, showed more potent G-CSF activity than that of intact human G-CSF *in vitro* and *in vivo* (5). Biological activity and receptor binding capacity were not lost at least 2 weeks after radioiodination of KW-2228 (9).

Preparation of Radioiodinated KW-2228. *Escherichia coli*-derived muteins G-CSF (KW-2228, more than 99% pure) (5) were radioiodinated with 0.5 mCi of Na^{125}I (Amersham, United Kingdom) using solid phase glucose oxidase-lactoperoxidase (10). In a 1.5-ml Eppendorf microtube, 10 μg of KW-2228 were added to 55 μl of 0.2 M sodium phosphate buffer (pH 7.2) followed by addition of 25 μl of 1% β-D-glucose (Sigma Chemical Co., St. Louis, MO), 10 μl of Enzymobead reagent (Bio-Rad Laboratories, Richmond, CA), and 5 μl (0.5 mCi) of Na^{125}I with vigorous stirring. Incubation was carried out for 1 h at room temperature. After incubation, the reaction was terminated by the addition of 25 μl of 5 mg/ml NaN_3 (Sigma). Radioiodinated KW-2228 was separated using 0.1 M sodium phosphate buffer (pH 7.0) containing 0.2% bovine serum albumin (Sigma) as an elution buffer. The specific activity of radioiodinated KW-2228 was approximately 2.0×10^8 cpm/μg protein determined by self-displacement analysis.

Cell Culture. All cell lines were maintained in RPMI 1640 (Flow Laboratories), supplemented with 10% fetal bovine serum and antibiotics. Cell lines used include the acute myelogenous leukemia cell line KG-1 (11), the human promyelocytic leukemia cell line HL-60 (12), chronic myelogenous leukemia cell line K-562 (13), B-lymphoid cell lines Namalwa (14) and Daudi (15), T-lymphoid cell line CCRF-CEM (16), and a histiocytic lymphoma cell line U-937 (17).

Preparation of Fresh Leukemia Cells. Heparinized peripheral blood was obtained from normal subjects or leukemia patients (7 patients with AML, 2 patients with ALL) at diagnosis, with informed consent. T-cell-depleted mononuclear cells from leukemia blood (AML) were separated using the two cycle Ficoll-Hypaque procedure, the second following the formation of E-rosettes (18). Leukemia cells from patients with ALL were separated by removal of adherent cells from the mononuclear cell fraction as described (19). These cells were used directly for binding studies or cultured in methylcellulose medium in the presence of G-CSF.

Colony Formation of Leukemic Blasts in Semisolid Culture. Leukemic cells (1×10^5) suspended in 0.5 ml of α-medium supplemented with 0.8% methylcellulose, 20% fetal bovine serum, and antibiotics were seeded in 24-well multiplates (Flow Laboratories) in the presence or the absence of human G-CSF (KW-2228). Incubations were carried out at 37°C in a humidified atmosphere of 5% CO_2 in air for 7 days. Cell aggregates with 20 cells or more were counted as colonies. The morphology of the cells in the colonies was examined microscopically after staining with Wright dye.

Binding Experiments. Unless otherwise stated, the binding experiments were performed at 15°C for 90 min in a total volume of 200 μl of α-medium containing 0.1% bovine serum albumin (Sigma), 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.01% bacitracin, 0.02% NaN_3 1×10^6 cells, and 100,000 cpm of ^{125}I-KW-2228 with or without a 50-fold excess of unlabeled KW-2228 in the test tubes. After incubation, the tubes were chilled on ice. The cells were resuspended and transferred onto 200 μl of di-*n*-butyl phthalate (Wako Pure Chem-

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[3] The abbreviations used are: G-CSF, granulocyte colony-stimulating factor; AML, acute myeloid leukemia; ALL, acute lymphoid leukemia.

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ical Ind., Ltd., Osaka, Japan) in 1.5-ml Eppendorf microtubes. After centrifugation for 5 min in a Beckman Microfuge B, the radioactivity of the pellets was counted in an Aloka Auto Well Gamma System (ARC-2511). The specific binding was determined as total binding minus binding in the presence of at least a 50-fold excess of unlabeled KW-2228.

Chemical Cross-Linking. Chemical cross-linking experiments were performed as described (20) with minor modifications. The cell pellet was resuspended in 0.5 ml of cold phosphate-buffered saline and incubated with disuccinimidyl suberate (Pierce Chemical Co., Rockford, IL) (final concentration of disuccinimidyl suberate, 0.9 mM) at 4°C for 15 min. The reaction was quenched by adding 1 ml of cold Tris-HCl buffer (10 mM, pH 7.4) with 1 ml of EDTA. The tubes were centrifuged, and the resultant pellet was solubilized in 40 µl of 25 mM Tris-HCl buffer (pH 7.4) containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (Sigma). After 10 min of incubation on ice, the tubes were centrifuged at 15,000 × g for 10 min at 4°C. The supernatant was collected and frozen at −70°C until use.

Electrophoresis and Autoradiography. Frozen cross-linked samples were thawed and mixed with 20 µl of 2-fold concentrated Laemmli's sample buffer (21) and then boiled for 5 min. Electrophoresis was performed according to the method of Laemmli (21). The sample was loaded onto 8% polyacrylamide/sodium dodecyl sulfate gels. The gels were stained (50% trichloroacetic acid-0.1% Coomassie blue), destained (7% acetic acid), dried, and autoradiographed using Kodak X-Omat AR film with Fuji EC-A Cassette (Fuji Photo Film Co., Ltd., Tokyo, Japan).

RESULTS

Binding of ^{125}I-KW-2228 to Human Leukemic Cell Lines. The specific binding of ^{125}I-KW-2228 to human leukemic cells increased dependently on the cell number. Representative data are shown in Fig. 1. The increase in the specific binding was linear up to 4 × 10^6 cells. We used 1 × 10^6 cells for the characterization of the specific binding.

The specific binding of ^{125}I-KW-2228 to human leukemic cells was both time and temperature dependent (Fig. 2). Higher specific binding was observed at 37°C than at 4°C or 15°C. Specific binding to the leukemic cells reached maximum for 60-min incubations.

The characterization of the specific binding of ^{125}I-KW-2228 to human leukemic cells was performed by Scatchard analysis (22). The Scatchard plot was linear, suggesting that a single G-CSF receptor type exists in human leukemic cells. The number of receptor per cell, calculated from the intercept of the Scatchard plot on the *abscissa* in Fig. 3, and the dissociation constant (K_d) in various human leukemic cells are summarized in Table 1. High affinity binding sites were identified in myeloid and monocytic cells but were absent in lymphoblastic cells (Namalwa, Daudi, or CCRF-CEM).

Binding of ^{125}I-KW-2228 to the Blast Cells of Myeloid Leukemia. Binding assays were done on fresh blasts within three-hours of separation on Ficoll-Hypaque. Scatchard analysis showed that a single binding site for G-CSF was observed. Representative data are shown in Fig. 4. The Scatchard plot was linear in all 7 AML blasts, suggesting that a single G-CSF

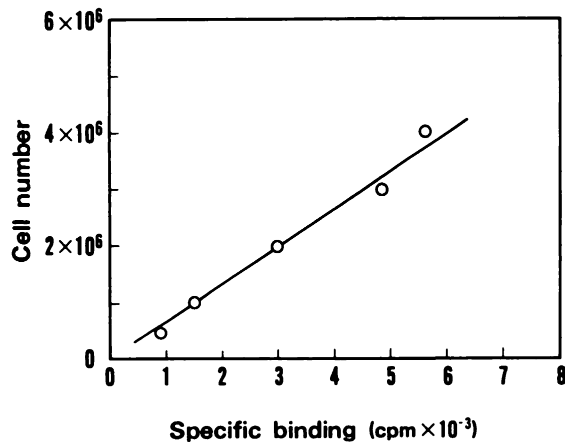


Fig. 1. Effect of cell number on the specific binding of ^{125}I-KW-2228 to U-937 cells (a human histiocytic lymphoma cell line). Binding experiments were performed as described in "Materials and Methods." The indicated number of cells was used.

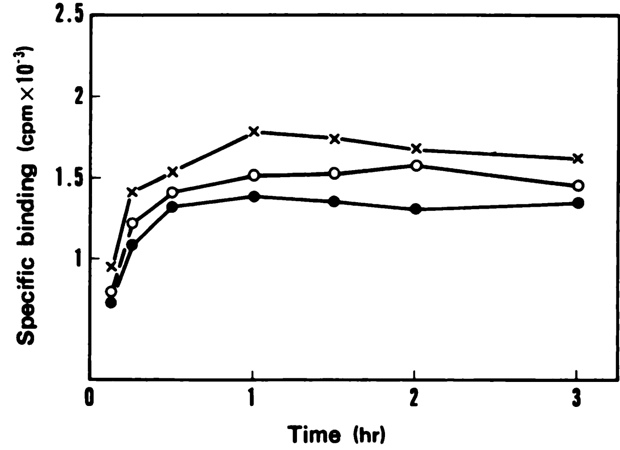
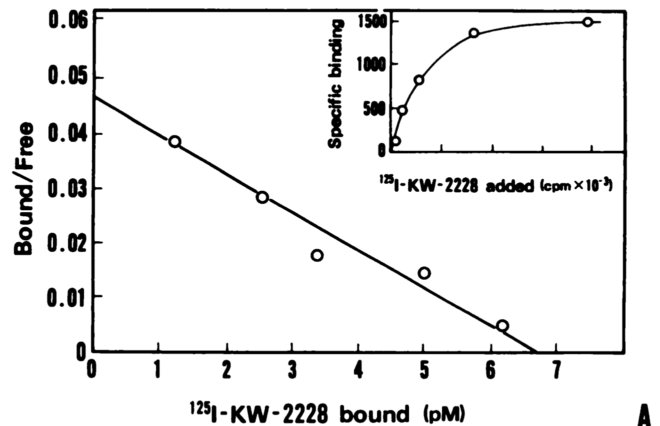


Fig. 2. Time and temperature dependency of the specific binding of ^{125}I-KW-2228 in U-937 cells. Incubations were performed for the indicated time at 4°C (●), 15°C (○), or 37°C (×) with 1 × 10^6 cells.



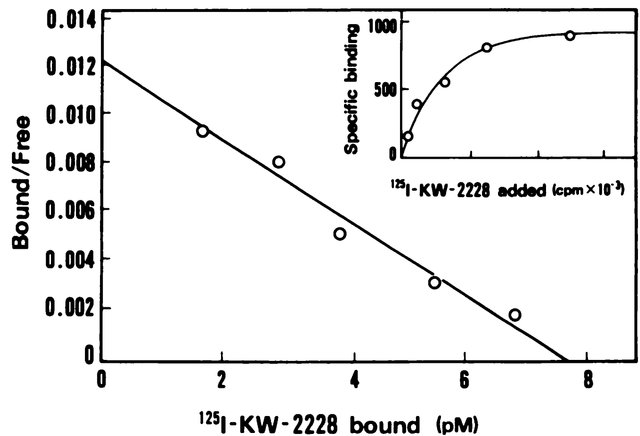


Fig. 3. Scatchard analysis of the specific binding data of ^{125}I-KW-2228 in human leukemia cell lines. Various concentrations of ^{125}I-KW-2228 were used. (*A*) U-937 cells; (*B*) KG-1 cells (1 × 10^6 cells).

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Table 1 *Binding parameters of ^{125}I-KW-2228 to the blast cells of patients with leukemia and cell lines*

	K_d (pM)	Binding sites/cell
Fresh blasts from		
Patient 1 (AML, M_2)	438	1686
Patient 2 (AML, M_1)	500	1324
Patient 3 (AML, M_3)	380	742
Patient 4 (AML, M_1)	128	1397
Patient 5 (AML, M_1)	750	361
Patient 6 (AML, M_2)	857	289
Patient 7 (AML, M_4)	1400	1688
Patient 8 (ALL)	0	0
Patient 9 (ALL)[a]	0	0
Patient 10 (ALL)	0	0
Patient 11 (ALL)	0	0
Patient 12 (ALL)	0	0
Cell line		
U-937	145	806
KG-1	570	824
HL-60	596	927
K562	400	361
Namalwa	0	0
Daudi	0	0
CCRF-CEM	0	0

[a] Patient 9 had B-cell lymphoma in leukemic stage.

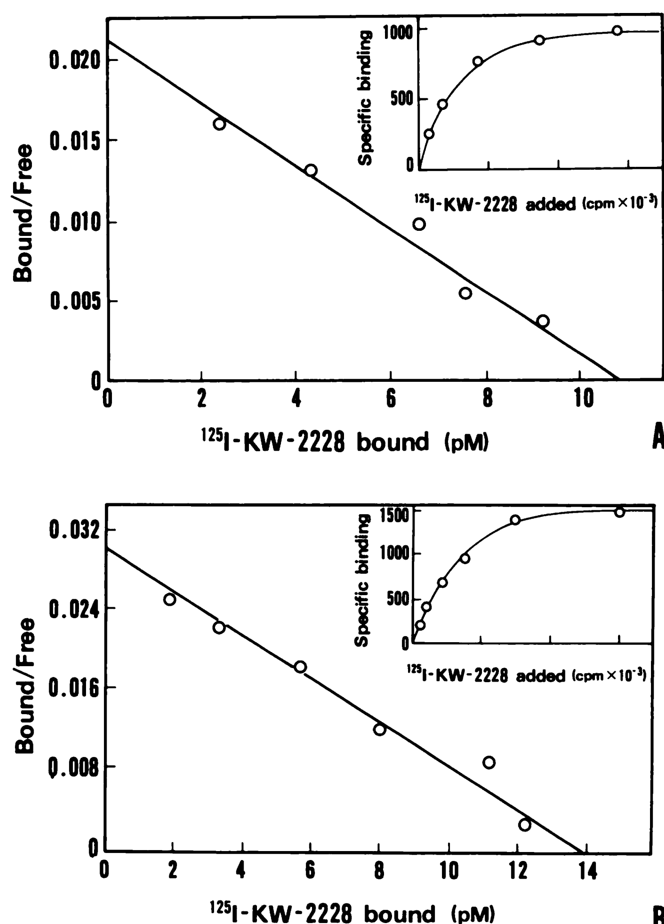

Fig. 4. Scatchard analysis of the specific binding data of ^{125}I-KW-2228 in the blast cells from patients with AML. Various concentrations of ^{125}I-KW-2228 were used. (*A*) blast cells from patient 2 (M_1); (*B*) blast cells from patient 1 (M_2). One × 10^6 cells were used.

receptor type exists in AML blasts. As in other biological parameters, patient-to-patient variation was marked. However, the number of receptors was similar to that on neutrophils (9) or leukemic cell lines (Table 1). Specific binding of ^{125}I-KW-2228 was not demonstrated in the blast populations obtained from patients with ALL or lymphoma.

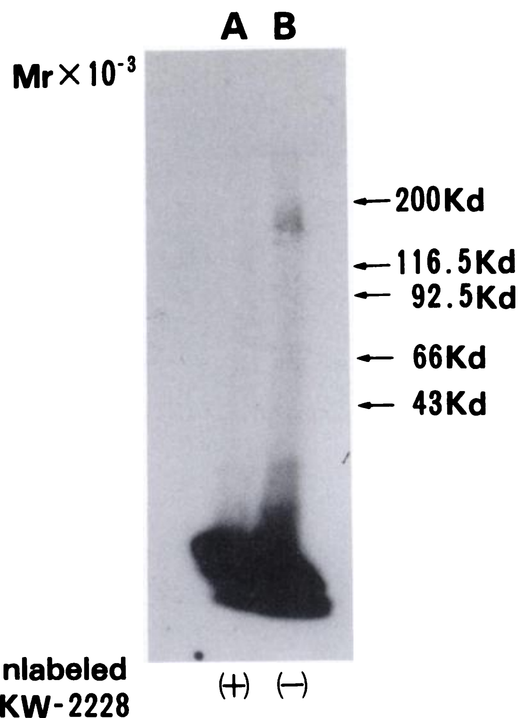

Fig. 5. Inhibition of the formation of cross-linked complex by unlabeled KW-2228. Chemical cross-linking was performed as described in "Materials and Methods." *Lane A*, in the presence of unlabeled KW-2228 (10 μg/ml); *Lane B*, in the absence of unlabeled KW-2228. K_d, molecular weight in thousands.

Chemical Cross-Linking. A representative result of the chemical cross-linking experiments is shown in Fig. 5. The autoradiography of ^{125}I-KW-2228 cross-linking to blast cells of myeloid leukemic showed a single radioactive band at a relative molecular mass of 170 kDa (Fig. 5, *Lane B*). This radioactive band disappeared in the presence of at least a 100-fold excess of unlabeled KW-2228 (Fig. 5, *Lane A*). The apparent molecular weight of the ^{125}I-KW-2228-receptor cross-linked complex was the same in reducing (Fig. 5) and nonreducing sodium dodecyl sulfate/polyacrylamide gels (data not shown).

Effect of KW-2228 on Colony Formation of Leukemic Blasts. To see whether KW-2228 stimulates the growth of leukemic blasts that have the specific receptor for KW-2228, we examined simultaneously the effect of KW-2228 on the *in vitro* colony formation by the blast cells obtained directly from the patients in Table 1. KW-2228 has been shown to stimulate the colony formation of leukemic blasts from all seven patients with AML (Fig. 6) but it was ineffective against the blasts of ALL or lymphoma (data not shown).

DISCUSSION

In this report, we described the existence of specific receptors for human G-CSF in human myeloid leukemic cells. A few researchers have reported that G-CSF receptors exist in murine bone marrow (23), human bone marrow (24), human neutrophils (25), and a few murine cell lines (25). However, these data were obtained using radiolabeled murine G-CSF. Binding data on G-CSF receptors using radiolabeled human G-CSF and human target cells have not been demonstrated. This may be due to the difficulty in obtaining radiolabeled human G-CSF that retains full biological activity and receptor binding capacity. Our mutein G-CSF, KW-2228, is more stable and potent than the intact human G-CSF (5). We compared the displacements of iodinated KW-2228 from neutrophils by either the

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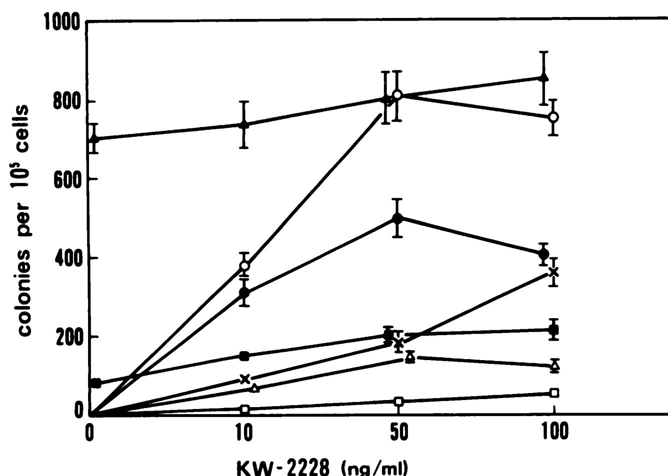

Fig. 6. Dose-response curves relating colony formation for AML blasts to KW-2228 concentration in methylcellulose. Patients 1 (△), 2 (●), 3 (×), 4 (○), 5 (▲), 6 (□), 7 (■).

KW-2228 protein or the intact G-CSF protein (9). KW-2228 was more efficient in displacing ^{125}I-KW-2228 than the intact G-CSF protein. The difference may reflect the higher biological activity of KW-2228. It retains full biological activity and receptor binding capacity even 2 weeks after radioiodination. We have demonstrated this with ^{125}I-KW-2228, a single receptor type in normal human neutrophils (560 receptors/cell, K_d 250 pM) (9). While patient-to-patient variation was marked as in other biological parameters, a single binding site for KW-2228 was also observed in myeloid leukemic blasts (Fig. 4; Table 1).

Chemical cross-linking data showed a single radioactive band which was lost by excess concentration of unlabeled KW-2228 (Fig. 5). From these data it can be concluded that the 170-kDa complex most probably represents ^{125}I-KW-2228 cross-linked to its specific receptor and, since ^{125}I-KW-2228 has an approximately molecular weight of 20,000, that the human G-CSF receptor on the blast cells of myeloid leukemia is a non-disulfide-bonded protein with an approximately molecular weight of 150,000. A similar molecular mass of G-CSF receptor for human neutrophils (9) and murine cell lines (26) was reported.

Whether the G-CSF receptor on the blast cells can serve as a useful marker to distinguish AML from ALL will have to be ascertained by further studies.

In Fig. 5, half-maximal stimulation of colony formation by KW-2228 was achieved at 0.4–1.7 nM in the blast cells of AML. Since half-maximal response to KW-2228 as measured by the stimulation of proliferation of normal progenitor cells occurs at about 5 pM, the extremely poor response of the blast cells of AML is intriguing. These results lead us to consider whether the G-CSF receptor or postreceptor system in the blast cells of AML exerts its function inefficiently. A considerable effort will be required to resolve this problem.

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