Generation and Characterization of Monoclonal Antibodies to Recombinant Human Granulocyte-colony Stimulating Factor (G-CSF) and Its Muteins

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Monoclonal antibodies (MoAbs) reacting with human G-CSF and/or its muteins were established by cell fusion between P3.X63/Ag8.U1 myeloma cells and spleen cells from BALB/C mice immunized with recombinant human intact G-CSF or its mutein, designated ND28. Two MoAbs reacted with intact G-CSF and all kinds of muteins tested, designated KM341 and KM342, and two MoAbs specific for intact G-CSF, designated KM340 and KM343, were obtained from the mice immunized with recombinant human intact G-CSF.

The sera from the mice immunized with a mutein of G-CSF, ND28, reacted with intact G-CSF and all muteins tested. Two MoAbs specific for ND28, designated KM498 and KM511, were obtained from these mice. These MoAbs seem to recognize the sequence of a few amino acids that is peculiar for ND28. However, the epitopes recognized by KM498 and KM511 were maybe subtly different, because KM498 and KM511 could not completely inhibit each other.

Human G-CSF and/or its muteins could be measured by sandwich ELISA using these MoAbs with suitable combinations. The immuno-affinity column using KM342 or KM498 adsorbed G-CSFs or specifically ND28, previously. By elution with 0.15 M NH₄OH, the G-CSFs or ND28 were eluted with a high recovery.

G-CSF acts as a physiological granulopoietin,^{1,2)} and is expected to be used for granulocytopenic recovery from granulocytopenia caused by radiation and administration of anti-cancer drugs. Since the cloning and sequencing of the human G-CSF gene,³⁾ recombinant DNA technology has produced recombinant human G-CSF^{1,4)} in sufficient amounts for clinical trials.

To find more effective G-CSF derivatives, many muteins of G-CSF were produced in *Escherichia coli* using recombinant DNA technology. One of them, designated ND-28, had two- to four-fold higher activity *in vitro* than intact G-CSF (personal communication).

For detection, quantification, and purification of G-CSF in industrial production and clinical studies, MoAbs specific for recombinant human G-CSF and/or ND-28, muteins of G-CSF were generated.

In this paper, we report the generation and characterization of the MoAbs against G-CSF and its muteins, and we describe their application to sandwich ELISA and to western blotting of G-CSF and its muteins.

Materials and Methods

G-CSF and its muteins. Recombinant intact human G-CSF, and its muteins, which have the amino acid se-

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Abbreviations: ELISA, enzyme linked immunosorbent assay; MoAb, monoclonal antibody; G-CSF, granulocyte-colony stimulating factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FCS, fetal calf serum; PBS, phosphate-buffered saline.

	1	3	4	5	7													17	
Intact:	Thr	Pro	Leu	Gly	Pro	Ala	Ser	Ser	Leu	Pro	Gln	Ser	Phe	Leu	Leu	Lys	Cys	...	
17 Ser:	Thr	Pro	Leu	Gly	Pro	Ala	Ser	Ser	Leu	Pro	Gln	Ser	Phe	Leu	Leu	Lys	Ser	...	
1 Ser :	Ser	Pro	Leu	Gly	Pro	Ala	Ser	Ser	Leu	Pro	Gln	Ser	Phe	Leu	Leu	Lys	Cys	...	
NC 59 :	Thr	Pro	Ile	Arg	Ser	Ala	Ser	Ser	Leu	Pro	Gln	Ser	Phe	Leu	Leu	Lys	Ser	...	
ND 28 :	Ala	Pro	Thr	Tyr	Arg	Ala	Ser	Ser	Leu	Pro	Gln	Ser	Phe	Leu	Leu	Lys	Ser	...	
NCD 28:	Ala	Pro	Thr	Tyr	Arg	Ala	Ser	Ser	Leu	Pro	Gln	Ser	Phe	Leu	Leu	Lys	Cys	...	
NC 95 :	Ile	Pro	Thr	Arg	Ser	Ala	Ser	Ser	Leu	Pro	Gln	Ser	Phe	Leu	Leu	Lys	Ser	...	
NCC 95:	Ile	Pro	Thr	Arg	Ser	Ala	Ser	Ser	Leu	Pro	Gln	Ser	Phe	Leu	Leu	Lys	Cys	...	
NC 93 :	Asn	Pro	Gln	Arg	Ser	Ala	Ser	Ser	Leu	Pro	Gln	Ser	Phe	Leu	Leu	Lys	Ser	...	
NCC 93:	Asn	Pro	Gln	Arg	Ser	Ala	Ser	Ser	Leu	Pro	Gln	Ser	Phe	Leu	Leu	Lys	Cys	...	
Arg 4 :	Thr	Pro	Leu	Arg	Pro	Ala	Ser	Ser	Leu	Pro	Gln	Ser	Phe	Leu	Leu	Lys	Cys	...	
Δ1-4S:					Pro	Ala	Ser	Ser	Leu	Pro	Gln	Ser	Phe	Leu	Leu	Lys	Ser	...	
M-7 :									Ser	Leu	Pro	Gln	Ser	Phe	Leu	Leu	Lys	Ser	...
M 7-2 :									Ser	Leu	Pro	Gln	Ser	Phe	Leu	Leu	Lys	Cys	...

Fig. 1. Sequence of Seventeen N-Terminal Amino Acids of Human Intact G-CSF and Its Muteins.

quences shown in Fig. 1, were produced and purified in our laboratories.

Immunization and fusion. Eight-week-old female BALB/c mice were immunized by *i.p.* injection of 100 μ g of the purified G-CSF or ND-28, a mutein of G-CSF, with 2 mg of aluminum gel and 10^9 cells of *Bordetella pertussis* as adjuvant, per head. Three or four consecutive injections of the same amount of the immunogen without adjuvant were given *i.p.* to the same mice at weekly intervals starting 2 weeks after the first immunization. The mice were killed 3 or 4 days after the last injection. The spleen cells were fused with P3.X 63/Ag8.U1 (P3U1), at a 5:1 ratio of spleen cells to P3U1, in PEG-1000 by a modification of the method of Köhler and Milstein.⁵⁾ The fused cells were distributed in 100 μ l samples into 96-well culture plates (Becton Dickinson Labware, Oxnard, U.S.A.) at a concentration of 10^5 spleen cells per well. The growth medium used consisted of RPMI-1640 (Nissui, Tokyo, Japan) containing 10% fetal calf serum. After three consecutive changes of the culture supernatant with a growth medium containing 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine, the antibody titer to G-CSF or ND-28 in the culture supernatant was measured by binding ELISA. Positive wells were subcloned twice by a limiting dilution method (one cell per three wells).

Binding ELISA. G-CSF or its mutein was distributed at

10 μ g per well into 96-well microtiter plates (Flow Labs. Inc., McLean, U.S.A.). After incubation at 4°C overnight or at 37°C for 2 hr, unbound materials were removed and 200 μ l of phosphate-buffered saline containing 1% (w/v) bovine serum albumin (BSA-PBS) was added to each well. The wells were washed three times with PBS after incubation under the conditions described above. One hundred microliters of MoAb or of the culture supernatant of a hybridoma clone was added to each well. The wells, which were incubated under the same conditions as above, were washed three times with PBS containing 0.05% Tween 20 (Tween-PBS). One hundred microliters of horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark) was then added to each well and the plates were incubated at 37°C for 2 hr. After washing five times with Tween-PBS, 100 μ l of peroxidase substrate solution [containing 0.05% 2,2'-azino-di-(3-ethylbenzothiazoline) sulfonic acid diammonium salt (ABTS; Nakarai, Kyoto, Japan), and 0.1% hydrogen peroxide in 0.1 M sodium citrate, pH 4.2] was added. After 1 min at room temperature, the reaction was stopped by the addition of 100 μ l of 5% SDS. The absorbance of each well was measured by microtiter plate analyzer (Japan Intermed, NJ-2000, Tokyo, Japan) at 415 nm using 490 nm as reference.

Preparation of MoAbs. The established hybridomas were propagated as ascites in Pristane (Aldrich Chemical

Co., Inc., Milwaukee, U.S.A.)-treated nude mice (BALB/c nu/nu).

A large amount of purified MoAbs were obtained from the ascites by ammonium sulfate precipitation, ion-exchange chromatography with DEAE-cellulose, DE-52 (Whatman, Clifton, U.S.A.), and affinity chromatography using an Affi-Gel Protein MAPS-II kit (Bio-Rad Laboratories, Richmond, U.S.A.). Part of the purified MoAbs were biotinylated by the method of Guesdon *et al.*⁶⁾ Briefly, purified MoAb was dialyzed against 0.1 M NaHCO₃ (pH 8.0) containing 0.5 M NaCl overnight at 4°C. One milliliter of dialyzed MoAb (1 mg/ml) was mixed with 100 μ l of *N*-hydroxysuccinimidobiotin (Sigma, St. Louis, U.S.A.) dissolved in *N,N'*-dimethyl formamide (1 mg/ml). The mixture was incubated for 4 hr at room temperature, and then was extensively dialyzed against 10 mM phosphate buffer (pH 7.2) containing 0.5 M NaCl.

Quantification of G-CSF and its muteins (Sandwich ELISA). For sandwich ELISA, wells of a 96-well EIA plate (Flow Laboratories, Inc., McLean, U.S.A.) were coated with 50 μ l of purified MoAb (10 μ g/ml) at 4°C overnight. After removal of the antibody solution, residual protein-binding sites on the plates were blocked with 100 μ l of 1% BSA-PBS. Then 50 μ l of samples containing G-CSF or its muteins at various concentrations were added to the wells and incubated overnight at 4°C. After these were washed with Tween-PBS, 50 μ l of avidin-biotin-peroxidase complex (ABC Kit; Vector, Burlingame, U.S.A.) was added and incubated at room temperature for 1 hr. Then the wells were washed with Tween-PBS and 50 μ l of ABTS solution was applied for 2 min at room temperature. The absorbance at 415 nm was measured.

SDS-PAGE and western blotting. SDS-PAGE was done in 10% (w/v) polyacrylamide gel by the method of Laemmli.⁷⁾ Two micrograms of G-CSF and its muteins were developed by SDS-PAGE, and they were transferred to nitrocellulose membranes in transfer buffer (0.025 M Tris, 0.2 M Glycine, 20% MetOH, pH 8.3) overnight at 4°C by the method of Towbin *et al.*⁸⁾ The dried nitrocellulose membrane was soaked in 3% gelatin solution to block the remaining protein-binding sites. Then it was dipped in culture supernatant of a hybridoma or in a solution of MoAb and incubated at room temperature for 2 hr. After being washed 3 times with Tween-PBS, peroxidase-labeled antimouse immunoglobulin was left to react at room temperature for 2 hr. After it was washed 3 times, the membrane was developed with 0.06% HRP Color Development Reagent (Bio-Rad) in 0.02 M Tris/0.5 M NaCl, pH 7.5. The reaction was stopped by soaking the membrane in cold water. Another membrane was stained with amido black after transfer.

Immuno-affinity chromatography. Purified MoAb was dialyzed against the coupling buffer (0.1 M NaHCO₃, 0.5 M

NaCl, pH 8.0) overnight at 4°C. Four milliliters of tressyl-activated Sepharose 4B gel (Pharmacia Fine Chemical, Uppsala, Sweden) washed and buffered with the coupling buffer was added to dialyzed antibody solutions and was mixed for 2 hr at room temperature. After this was washed with phosphate buffer containing 0.5 M NaCl, one-half milliliter of gel was poured into the column and equilibrated at 4°C with phosphate buffer containing 0.5 M NaCl.

Two hundred micrograms of partially purified MoAb were put onto the thus-prepared immunoaffinity column. After washing the column with phosphate buffer containing 0.5 M NaCl, adsorbed components were eluted with 0.15 M NH₄OH. Protein in each 1-ml fraction was measured by the method of Lowry *et al.*,⁹⁾ and G-CSF or ND-28 contents by the sandwich ELISA described above. Before ELISA, the eluted fractions were dialyzed against phosphate buffer containing 0.5 M NaCl.

Results

Generation of MoAbs against G-CSF or ND-28

Two 8-week-old female BALB/c mice were immunized by recombinant human intact G-CSF and five mice were immunized by ND-28, a mutein of G-CSF. The sera from all mice reacted with both intact G-CSF and ND-28, but not to bovine serum albumin (BSA) and pKYP-10 (admixed proteins from host *Escherichia coli*). (Data not shown.)

Finally, four MoAbs, designated KM340, KM341, KM342, and KM343 from the mice immunized intact G-CSF, and two MoAbs, designated KM498 and KM511, from the mice immunized with ND28 were selected for their reactivity to G-CSF and/or its muteins. The isotypes of these MoAbs were identified by the double diffusion method. All MoAbs were IgG₁.

Binding specificities of MoAbs

The reactivities by ELISA of KM340, KM341, KM342, and KM343 to various recombinant human proteins are shown in Fig. 2. KM342 reacted weakly with r-hu-interferon- γ (IFN- γ) and r-hu-interferon- β (IFN- β). KM340, KM341, and KM343 reacted only to r-hu-G-CSF. This specificity of these MoAbs to r-hu-G-CSF was confirmed by western blotting (Fig. 3).

Table I shows the specificities of MoAbs which were examined by binding ELISA using

various mutants of G-CSF. KM341 and KM342 reacted with intact G-CSF and all mutants of G-CSF tested. KM340 and KM343

reacted with intact, 17-Ser, 1-Ser, and Arg4. KM498 and KM511 reacted with only ND28 and NCD28.

In western blotting, KM498 gave only the band of ND28 (Fig. 4).

These results suggested that KM340 and KM343, KM341, and KM342, and KM498 and KM511 recognized the N-terminal several amino acid sequence of intact hu-G-CSF, the C-terminal amino acid sequence behind the 7th position of intact hu-G-CSF, and the N-terminal several amino acid sequence of ND28, respectively. As shown in Fig. 5, KM498 and KM511 did not completely inhibit the binding to ND28 of each other. It seems that the epitopes of KM498 and KM511 are different.

Fig. 2. Reactivity of MoAbs Derived from the Mice Immunized with Recombinant G-CSF to Various Recombinant Proteins in Binding ELISA.

A), anti-human G-CSF mouse serum; B), KM340; C), KM341; D), KM342; and E), KM343.

Antigens: 1, intact G-CSF; 2, human interferon- γ ; 3, human interferon- β ; 4, human interleukin-1; 5, human interleukin-2; 6, admixture of proteins prepared from host *Escherichia coli*.

Quantification of G-CSFs by sandwich-type ELISA

Quantification of intact G-CSF and/or ND28 by sandwich-type ELISA were examined. The titration curve of intact G-CSF by sandwich-type ELISA using KM340 and biotinylated KM341 is shown in Fig. 6. It was demonstrated that in the range of 2.5~80 ng/ml intact G-CSF could be quantified by this assay system. ND28 was not identified in this sandwich-type ELISA.

Figure 7 shows a titration curve of intact G-CSF and ND28 by sandwich-type ELISA

Fig. 3. Specificity of Anti-G-CSF MoAbs in Western Blotting.

1, human interferon- γ ; 2, human interferon- β ; 3, intact G-CSF; 4, MW markers were developed by SDS-PAGE and transferred to nitrocellulose membranes. After transfer, one membrane was stained with amido black, A), and the others were stained with KM340, B), KM341, C), KM342, D) and KM343, E).

Table I. REACTIVITY OF ANTI-G-CSFs MoAbs TO INTACT G-CSF AND ITS MUTEINS

Antigen	Monoclonal antibody					
	KM340	KM341	KM342	KM343	KM498	KM511
Intact	○	○	○	○	×	×
17 Ser	○	○	○	○	ND	ND
1 Ser	○	○	○	○	×	×
NC 59	×	○	○	×	×	×
ND 28	×	○	○	×	○	○
NCD 28	×	○	○	×	○	○
NC 95	×	○	○	×	ND	ND
NCC 95	×	○	○	×	ND	ND
NC 93	×	○	○	×	×	×
NCC 93	×	○	○	×	ND	ND
Arg 4	○	○	○	○	ND	ND
$\Delta 1 \sim 4S$	ND	ND	ND	ND	×	×
M-7	ND	ND	ND	ND	×	×
M-7-2	×	○	○	×	ND	ND

Reaction: ○, positive; ×, negative.

ND, not done.

Fig. 4. Specificity of KM498 in Western Blotting.

①, MW markers; ②, intact G-CSF; ③, ND28; ④, admixture of proteins prepared from host *E. coli*, were developed by SDS-PAGE and transferred to nitrocellulose membranes.

After transfer, one was stained with amido black, A), and others were stained with anti-ND28 mouse serum, B) and KM498, a anti-ND28 MoAb, C), respectively.

using KM341 and biotinylated rabbit anti-G-CSF polyclonal antibody. This assay system allows measurement of intact G-CSF and ND28 of concentrations from 2 to 10 ng/ml.

The value of OD_{415} of sandwich-type ELISA using KM498 and biotinylated KM341 corresponded well to the concentrations of ND28 between 0.05~10 $\mu\text{g/ml}$ (Fig. 8).

In this system intact G-CSF was not iden-

tified.

Purifications of G-CSFs by affinity chromatography

Intact G-CSF solution (62.7 $\mu\text{g/ml}$) was put on the KM342 coupled Sepharose 4B column which was prepared as described in **Materials and Methods**.

Unbound materials having no antigenic ac-

Fig. 5. Inhibition of Binding Activity to ND28 of Biotinylated KM498 and Biotinylated KM511 by KM511 and KM498.

Fifty μ l of the mixture of the biotinylated MoAbs (A, biotinylated KM498; B, biotinylated KM511) at the concentration of 20 μ g/ml and various concentrations of native MoAb (inhibitors: $\circ$, KM498; $\bullet$, KM511) were added to each well of a 96-well EIA plate which was coated beforehand with ND28 and incubated at room temperature for 2 hr. After washing with 0.05% Tween 20 in PBS, 50 μ l of avidin-peroxidase (2.5 μ g/ml) were added at room temperature for 1 hr. Enzyme activity was measured as described in **Materials and Methods** using ABTS as a substrate.

Fig. 6. Titration Curve of Intact G-CSF by Sandwich ELISA Using KM340 and Biotinylated KM341.

Various concentrations of intact G-CSF ($\bullet$), and ND28, a mutein of G-CSF ($\circ$) were measured by sandwich ELISA using KM340 and biotinylated KM341.

tivity effused in the first several fractions. After washing, G-CSF was eluted as a single peak with 0.15 M NH_4OH . This eluted G-CSF was a single protein containing impurities by SDS-PAGE (data not shown). In the purification

Fig. 7. Titration Curve of Intact G-CSF and ND28 by Sandwich ELISA Using KM341 and Biotinylated Rabbit Anti-G-CSF Polyclonal Antibody.

Various concentrations of intact G-CSF ($\bullet$), and ND28 ($\circ$) were measured by sandwich ELISA using KM341 and biotinylated rabbit anti-G-CSF polyclonal antibody.

Fig. 8. Titration Curve of ND28 by Sandwich ELISA Using KM498 and Biotinylated KM341.

Various concentrations of intact G-CSF ($\bullet$), and ND28 ($\circ$) were measured by sandwich ELISA using KM498 and biotinylated KM341.

of G-CSF, affinity chromatography using KM342 is considered to be superior to the conventional method which needs many steps and has a low yield.

By the affinity chromatography using KM498, a MoAb specific to ND28, the efficient purification of ND28 similarly was performed.

Discussion

Several MoAbs were established from the

mice immunized with human G-CSF and its mutein produced by recombinant DNA technology. Two MoAbs, KM341 and KM342, derived from the mice immunized with intact G-CSF reacted with intact G-CSF and all kinds of its muteins tested. These MoAbs seem to recognize the epitope in the C-terminal behind position 7. The other two MoAbs derived from mice immunized with intact G-CSF, KM340 and KM343, reacted with only intact G-CSF, 1-Ser, and 17-Ser. The epitope is a sequence of several amino acids between positions 2 to 6 of intact G-CSF. The antiserum prepared from the mice immunized with ND28, a mutein of human G-CSF, reacted with intact G-CSF and all of the muteins tested. Two MoAbs derived from the mice, KM498 and KM511, reacted specifically with only ND28 and NCD28. KM498 and KM511 seem to recognize the sequence including a few amino acids peculiar to ND28 and NCD28. The epitopes of KM498 and KM511 may not be quite the same, because KM498 and KM511 were not able to completely inhibit each other.

These anti-human G-CSFs MoAbs are useful for detection, quantification (monitoring), and purification of G-CSF and/or its muteins.

Further studies are in progress to test the relation of the epitopes and the G-CSF activity using these anti-G-CSFs MoAbs.

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