A Role for the Carboxyl Terminus of Human Granulocyte-Macrophage Colony-stimulating Factor in the Binding of Ligand to the α-Subunit of the High Affinity Receptor*

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A synthetic segment (110–127) of the carboxyl terminus of recombinant human granulocyte-macrophage colony-stimulating factor (rh-GM-CSF) was used to generate a rabbit polyclonal antibody (345-6), which recognized both peptide and full-length Escherichia coli-derived rh-GM-CSF in a direct enzyme-linked immunosorbent assay. Antibody 345-6 was shown to antagonize the binding of 125I-labeled rh-GM-CSF to its receptor on the KG-1 cell line and to inhibit human GM-CSF-dependent proliferation of the AML-193 cell line. The purified IgG fraction of neutralizing antibody 345-6 was used as immunogen to obtain sheep antiserum 1418. Antibody 1418 recognized antibody 345-6 on direct enzyme-linked immunosorbent assay but did not recognize rh-GM-CSF or the peptide 110–127 to which antibody 345-6 was raised. Antiserum 1418, as well as a purified IgG fraction of this serum, inhibited both rh-GM-CSF-stimulated cell proliferation and 125I-labeled rh-GM-CSF receptor binding but not 125I-labeled recombinant human interleukin-4 receptor binding. The anti-idiotypic antibody response derived from the anti-(110–127) antibody strongly suggests that the carboxyl-terminal region of rh-GM-CSF may directly be involved in the receptor-ligand interaction of this protein. The high affinity receptor consists of two different components (GM-α) a cytokine-specific α-subunit and a β-subunit that is shared by human GM-CSF, interleukin-3, and interleukin-5. In an effort to localize the epitope of antibody 1418 to either GMα or GMβ, several cell lines containing high, low, or both high and low affinity receptors were examined. Each was specifically and completely inhibited by antibody 1418. Interleukin-3-dependent cell proliferation of the AML-193 cell line was found to be unaffected by the antibody 1418. Thus, the carboxyl-terminal region of rh-GM-CSF is likely to be involved in the interaction of the ligand with the α-subunit of the high affinity receptor.

Human granulocyte-macrophage colony-stimulating factor (h-GM-CSF) is a member of a family of glycoproteins that stimulates growth and differentiation of a number of cell types of hematopoietic lineage (Metcalf, 1986; Clark and Kamen, 1987). Clinical trials with rh-GM-CSF derived from mammalian and bacterial hosts (Greenberg et al., 1988; Trotta, 1989) have been initiated in a variety of disease states, including acquired immunodeficiency syndrome (Groopman et al., 1987; Baldwin et al., 1988), myelodysplastic syndrome (Vadan-Raj et al., 1987), bone marrow transplantation (Monroy et al., 1987), and in combination with chemotherapy in cancer patients (Brandt et al., 1988).

The actions of h-GM-CSF are mediated through binding to cell-surface receptors (Gasson et al., 1986; DiasPersio et al., 1988). The high affinity receptor is believed to be a heterodimer consisting of both a cytokine-specific α-subunit and a β-subunit that is shared with IL-3 and IL-5 (Hayashida et al., 1990). The α-subunit of the receptor binds to the h-GM-CSF with low affinity (Gearing et al., 1989). Formation of the high affinity receptor occurs only in the complex of h-GM-CSF with both the α- and β-subunits. While the β-subunit does not itself bind to h-GM-CSF in the absence of the α-subunit, it does appear to interact directly with h-GM-CSF in the complex (Shanafelt et al., 1991a).

Significant insight to the specific residues on mouse GM-CSF in contact with the β-subunit of the receptor in the high affinity receptor-ligand complex has been provided by Shanafelt et al. (1991b) and by Shanafelt and Kastelein (1992). In addition, critical cytoplasmic domains of the β-receptor for growth signal transduction and tyrosine phosphorylation have been elucidated by Sakamaki et al. (1992). The residues on h-GM-CSF that may be involved in the binding of the α-subunit of the high affinity receptor are not yet known. In the present study, we sought to determine the domains of rh-GM-CSF that are involved in receptor interaction by raising both polyclonal antibodies to a synthetic peptide fragment and raising anti-idiotypic antibodies. The results support the conclusion that there is a segment at the carboxyl terminus of rh-GM-CSF that interacts directly with the cell-surface receptor. Further, the evidence provided suggests that it is likely to be the α-subunit of the receptor that binds to this terminal segment of h-GM-CSF.

EXPERIMENTAL PROCEDURES

Materials—Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD) and Jackson Immunoresearch Laboratories (Avondale, PA) supplied horseradish peroxidase-conjugated goat anti-rabbit and donkey anti-sheep IgG, respectively. Calbiochem and DuPont NEN supplied monoclonal antibodies VM-C6 and VM-2 and 125I-labeled Bolton-Hunter reagent (2000 Ci/mmol), respectively.

Cell Cultures and Reagents—The KG-1 cell line was obtained from the ATCC (CCL 246), and the AML-193 and TF-1 cell lines were provided to Schering-Plough Research Institute by Dr. G. Rovera (Lange et al., 1987) and Dr. T. Kitamura of DNAX Research Institute, respectively. Placental membranes were prepared by homogenization in the presence of protease inhibitors, centrifugation to remove the
100 x g fraction, and thorough washing of the 27,300 x g fraction.

Cytokines—rh-GM-CSF (Escherichia coli, nonglycosylated; Schering-Plough/Sandoz) was purified to a constant maximal specific activity by methodology similar to that described for recombinant human IL-3 and purified Chinese hamster ovary cell-derived recombinant human IL-4 are expressed in the laboratories of the Schering-Plough Research Institute.

Preparation of Radiolabeled rh-GM-CSF—rh-GM-CSF was radiolabeled by the method of Bolton and Hunter (1975) and purified by gel filtration on a Sephadex G-25 column (PD-10, Pharmacia LKB Biotechnology Inc.). The resulting 125I-labeled rh-GM-CSF had a specific radioactivity of 1-3 x 105 cpm/μg and a stoichiometry of 0.4-1.2 mol of 125I/mol of rh-GM-CSF. The specific radioactivity and stoichiometry were determined by the self-displacement column method (Calvo et al., 1983). 125I-labeled rh-GM-CSF exhibited the same level of biological activity in KG-1 and AML-193 cell proliferation assays as unlabeled GM-CSF.

Peptide Synthesis—The synthesis of peptides was carried out with an Applied Biosystems (Foster City, CA) model 430A fully automated peptide synthesizer (Merrifield, 1963). The amino-terminal tert-bu- tyloxyacarbonyl group was removed with 95% trifluoroacetic acid and cleaved from the phenylacetimido (polystyrene) resin with a 10:1.5 ratio of liquid hydrogen fluoride- anisole at 0 °C for 60 min. Cleaved, deprotected peptides were purified by reversed-phase high performance liquid chromatography on a C-4 Dynamax 40 A wide bore column (Rainin Assoc., Woburn, MA). Automated sequencing and fast atom bombardment mass spectral analysis were employed to confirm the peptide sequence.

Production and Purification of Antibodies—Rabbits were injected intramuscularly with 2 μg of antigen in 400 μl of TBS and 100 μl of pertussis vaccine and 500 μl of Freund's complete adjuvant. Boosts with incomplete adjuvant were scheduled when bleeds (as judged by ELISA) showed any loss of titer. The polyclonal antibody 1418 was produced in sheep by subcutaneous injection with 2.5 mg of pertussis vaccine and 25 μg of rabbit antisera 345-6. Sheep antibody 1418 was purified by Fortran Biotechnology Co. (Saint Mary's, PA) by ammonium sulfate precipitation and QAE ion exchange chromatography.

ELISA—Rabbit and sheep sera were screened for specific binding of antibodies by coating a 96-well microtiter plate (Becton-Dickinson) with 100 μl of antigen for 1 h at room temperature. Plates were washed with TBS containing 0.05% Tween 20 (TBST), blocked with 1% bovine serum albumin for 1 h, washed with TBST, blocked with 0.1% immunoglobulin for 1 h, and then washed with TBST. Blocking with immunoglobulin was omitted from the procedure for antibody 1418. The wells were coated with the antibody to be tested for 2 h, washed with TBST, then coated with 2.5 μg of horseradish peroxi- dase-conjugated goat anti-rabbit IgG or 5.0 μg of donkey anti-sheep IgG for 1 h, and then washed with TBST. Development with either 2.2'-azino-bis(3-ethyl-benzthiazoline sulfonate) or 3,3',5',5'-tetramethyl benzidine and hydrogen peroxide was detected colorimetrically 20 min later. Control wells were developed in one of which the three assay components (i.e. antigen, antibody, peroxidase-labeled antibody) was deleted.

Receptor Binding Assay—Assays to measure 125I-labeled rh-GM-CSF binding to receptors on KG-1 and AML-193 cells contained 50-100 μg 125I-labeled rh-GM-CSF, 4-6 x 106 cells, and IMDM containing 10% FCS in a total volume of 0.4 ml. Samples were incubated at 4 °C for 2 h and centrifuged for 2.5 min at 600 x g, and the cell pellet was washed twice with IMDM, 10% FCS and counted in a γ counter. Binding of 125I-labeled rh-GM-CSF to receptors on placental membranes was quantitated by incubating membranes for 1 h at 22 °C with 0.5-5.0 nM 125I-labeled rh-GM-CSF and IMDM, 10% FCS in a total volume of 0.4 ml, centrifuging for 2.5 min at 8000 g, washing the pellet with IMDM, 10% FCS, and counting. Saturation concentrations of unlabeled rh-GM-CSF were assayed with each antibody. The data are presented as specific binding, which was determined by subtracting nonspecific from total 125I-labeled rh-GM-CSF bound.

Competitive displacement of 125I-labeled rh-GM-CSF from receptors by peptides or polyclonal antibodies was measured by including the following concentrations of unlabeled antibodies. The ratio of 125I-labeled GM-CSF were incubated for 10 min at 4 °C with 125I-labeled rh-GM-CSF prior to initiating binding by the addition of cells. Peptides and the sheep polyclonal antibody 1418 were incubated for 10 min at 4 °C with cells before 125I-labeled rh-GM-CSF addition. Corresponding preimmune serum was also examined in control assays. None of the rabbit or sheep preimmune sera (or purified commercially available rabbit or sheep IgG) interfered with GM-CSF receptor binding.

Cell Proliferation Assay—The assay for GM-CSF is based on stimulation of proliferation of KG-1 or AML-193 cells. KG-1 is a cell line established from the bone marrow of a patient with AML (Lusis and Koeffler, 1980; Koeffler and Golde, 1978). The AML-193 is an acute childhood leukemia-derived cell line, which is dependent upon GM-CSF to support continued growth in vitro (Lange et al., 1987). The TF-1 is a cell line established from a patient with erythroleukemia, which shows complete dependence on GM-CSF or IL-3 to support sustained growth (Kitamura et al., 1989). Approximately 1 x 106 cells in IMDM containing 5 mg/ml each of insulin, transferrin, and sodium selenite were incubated in microtiter plate wells with dilutions of rh-GM-CSF for 6 days at 37 °C and then incubated for an additional 4 h with the tetrazolium salt 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide (Mossman, 1980). The results are expressed as a change in optical density (proportional to the log cell concentration), which represents the difference in optical density of the sample and a base-line control lacking rh-GM-CSF.

Protein Concentration—Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard, unless otherwise noted.

RESULTS

Inhibition of Receptor Binding and Biological Activities by Anti-peptide Polyclonal Antibodies—In order to probe the regions of rh-GM-CSF that may be important to its receptor binding and biological activities, a series of peptides were synthesized (Table I). Rabbit polyclonal antibodies raised against each of these peptides were examined for their ability to bind peptide and rh-GM-CSF and to prevent binding of rh-GM-CSF to its receptor on KG-1 or AML-193 cells. Anti-serum from one of these, anti-(110-127) antibody (Ab 345-6), shown by direct ELISA to bind both peptide 110-127 and rh- GM-CSF, was determined to completely inhibit receptor binding. In contrast, of the five other antisera that recognized (by ELISA) both the peptide to which they were raised and rh- GM-CSF, none blocked binding of rh-GM-CSF to receptor. Based on these data, we sought to further explore the role that this carboxyl-terminal segment might have in the actions of rh-GM-CSF.

In order to determine whether receptor and biological activities were specifically affected by this antibody, we purified the antiserum Ab 345-6 from rabbits by chromatography. The charged IgG blocked specific binding of 125I-labeled rh-GM-CSF to its receptor on KG-1 cells in a concentration-dependent manner. Binding was completely prevented by preincubation of 125I-labeled rh-GM-CSF with approximately 1.0 μg/ml of purified Ab 345-6 IgG. Half-maximal inhibition could be obtained using a concentration of 0.06 μg/ml (Fig. 1A). Nonimmunized rabbit serum and purified IgG had no effect on binding or nonspecific binding, respectively.

Next, the ability of purified Ab 345-6 to block rh-GM-CSF-stimulated proliferation of the AML-193 cell line was probed (Fig. 1B). Inhibition of proliferation was concentration-dependent, and 75% of rh-GM-CSF-dependent growth was prevented at 0.5 μg/ml IgG (the highest concentration examined). Half-maximal inhibition was obtained at 0.1 μg/ml in good agreement with the receptor binding inhibition observed. In the absence of rh-GM-CSF, no stimulation of AML-193 cell growth was observed. In addition, in the absence of rh-GM-CSF and the presence of sufficient quantities of Ab 345-6 to completely block rh-GM-CSF-stimulated proliferation of the AML-193 cell line, no change was observed in the AML-193 cell growth. Similarly, Ab 345-6 is shown to block rh- GM-CSF-stimulated proliferation of the TF-1 cell line under similar conditions (data not shown).

Inhibition of rh-GM-CSF by Anti-idiotypic Antibodies—We sought to determine whether the carboxyl terminus of rh-
**TABLE I**

| Antibody | Sequence no. | Sequence | Peptide response | Protein response | Inhibition |
|----------|--------------|----------|-----------------|-----------------|------------|
| 347-6    | 7-25 Cys     | PSPSTQPWEHVNAIQEARRLC | 90,000 | 90,000 | – |
| 346-6    | Cys 26-41    | CLNLSRTDAEMNETVE | 8,000 | 8,000 | – |
| 351-6    | 45-54        | EMFDLQEPITC | 10,000 | 10,000 | – |
| 171-87   | 54-67        | CLQTRLELYQGGLR | 5,000 | 0 | – |
| 177-87   | 74-92        | KGPLTMASHYKQHCPPTP | 100,000 | 0 | – |
| 137-88   | 94-119       | TSCATQITFESFKENLK | 10,000 | 10,000 | – |
| 172-87   | 96-111       | CATQITFESFKENLK | 1,000 | 1,000 | – |
| 345-6    | 110-127      | LKDFLLVIFDCEWPVQE | 10,000 | 10,000 | + |
| 349-6    | GM-CSF       | NA       | 90,000 | 90,000 | + |

* Recognition of peptide in a direct ELISA. Dilution gives 50% absorbance maximum. ELISA is coated with 0.25 pg/well of peptide or protein. No response was observed for preimmune serum.

**GM-CSF could play a role in the ligand-receptor binding site of this cytokine. Peptide 110-127 itself was not an effective antagonist of rh-GM-CSF binding to KG-1 cells or agonist of rh-GM-CSF-independent cell proliferation of AML-193 cells (concentrations 100-fold greater than the concentration of rh-GM-CSF were required to displace 50% of the radiolabeled ligand or stimulate proliferation). Therefore, we decided to explore alternative approaches to determine the role of the carboxyl terminus in the ligand-receptor binding site rh-GM-CSF. We chose to determine whether an anti-idiotypic antibody response could be generated using the polyclonal anti-110-127 antibody. Purified Ab 345-6 IgG was used to immunize a sheep, and the antiserum obtained (Ab 1418) recognized the purified Ab 345-6 but not peptide 110-127 or rh-GM-CSF (Fig. 2).

Antiserum Ab 1418 was then tested for its ability to inhibit binding of 125I-labeled rh-GM-CSF to AML-193 cells. The dose-dependent inhibition of binding by Ab 1418 is shown in Fig. 3A. Under the same conditions, preimmune serum had no effect. Ab 1418 had no effect on the nonspecific binding of 125I-labeled rh-GM-CSF to the cells. The specificity of the interaction is further supported by evidence that demonstrates that Ab 1418 had no effect on the binding of 125I-labeled IL-4 to receptors on Daudi cells (data not shown). Unpurified Ab 1418 was also tested for its ability to inhibit binding of 125I-labeled rh-GM-CSF to KG-1 cells. A dose-
dependent inhibition curve for KG-1 cells was obtained, which was similar to that shown in Fig. 3A for AML-193 cells.

The ability of Ab 1418 to inhibit the rh-GM-CSF-dependent proliferation of AML-193 cells was examined using purified Ab 1418 IgG. Dose-dependent inhibition was obtained with complete inhibition being achieved with an IgG concentration of 1 mg/ml (Fig. 3B). Purified anti-idiotypic antibody Ab 1418 IgG was not able to stimulate proliferation of AML-193 even at suboptimal concentrations of rh-GM-CSF. It was also unable to stimulate AML-193 proliferation in the absence of added rh-GM-CSF. In similar experiments conducted with the KG-1 cell line, Ab 1418 serum was also shown to inhibit rh-GM-CSF-dependent proliferation of the KG-1 cells in a dose-dependent manner (data not shown). Similar observations were made for the inhibition of rh-GM-CSF-stimulated proliferation of the TF-1 cell line by purified AB 1418.

**Characterization of Neutralizing Anti-idiotypic Antibody 1418**—The anti-idiotypic response obtained from the anti-110–127 antibody suggests that this antibody possesses the "internal image" and, thus, recognizes the rh-GM-CSF receptor. This receptor is believed to be a heterodimer consisting of an α-subunit, which is cytokine-specific, and a β-subunit, which is required for formation of the high affinity receptor. We wished to see if the epitope of the anti-idiotypic antibody Ab 1418 could be localized to the α- or β-subunit domains. We first attempted to see if Ab 1418 could inhibit the high and low affinity receptor activities. We hoped to distinguish the two by examining cell lines and receptor preparations that exhibit the low affinity α receptor and the high affinity α/β receptor (Table II). The KG-1 and AML-193 cell lines each display the high affinity receptor binding sites. While the KG-1 cell line also exhibits the low affinity receptor binding sites, no low affinity receptor binding site is observed for the AML-193 cell line. In contrast, both placental membranes and the choriocarcinoma-derived JAR cell line display only the low affinity receptor binding sites. These cell lines and membrane preparations, which appear to have high affinity sites, high and low affinity sites, or just low affinity sites are all completely and specifically inhibited by 1.4 mg/ml purified Ab 1418 IgG. This suggests that the α-subunit (GMRA) contains the Ab 1418 epitope, and, therefore, the
The high affinity receptor of h-GM-CSF heterodimer consists of the α-subunit, which confers cytokine specificity, and the β-subunit. The α-subunit alone displays low affinity for GM-CSF. The high affinity is displayed only when the receptor subunits of h-GM-CSF are each heterodimers. In each case, the α-subunit is distinct and specific for the cytokine. The β-subunit, however, which is essential for signal transduction. We demonstrate that the anti-idiotype antibody Ab 1418 inhibits binding of rh-GM-CSF to both the high and low affinity receptor binding sites of several cell lines. This strongly indicates that the epitope for this antibody lies on the α-subunit. The fact that the Ab 1418 was derived from an antibody directed to a peptide corresponding to amino acids 110–127 of h-GM-CSF is involved in the GMRe interaction.

We then wanted to confirm that Ab 1418 was targeting the α-subunit (GMRe). We chose to examine whether Ab 1418 would inhibit the IL-3-dependent proliferation of AML-193 cells. IL-3 shares the β-subunit (GMRe) with rh-GM-CSF. No inhibition of this biological activity was observed, suggesting that the β receptor subunit was not effected by this antibody and confirming that the involvement was occurring at the α-subunit.

**DISCUSSION**

In this study, we provide evidence for the importance of the carboxy-terminal 18 amino acid residues of rh-GM-CSF in binding of the ligand to its cell-surface receptor and expression of biological activity. The anti-idiotype antibody response underlines the importance of the carboxyl region and further suggests that this segment plays a direct role in the ligand-receptor binding site of rh-GM-CSF.

The high affinity receptor of h-GM-CSF heterodimer consists of the α-subunit, which confers cytokine specificity, and the β-subunit. The α-subunit alone displays low affinity for GM-CSF. The high affinity is displayed only when the β-subunit is also present. Both subunits are required for signal transduction. We demonstrate that the anti-idiotype antibody Ab 1418 inhibits binding of rh-GM-CSF to both the high and low affinity receptor binding sites of several cell lines. This strongly indicates that the epitope for this antibody lies on the α-subunit. The fact that the Ab 1418 was derived from an antibody directed to a peptide corresponding to amino acids 110–127 of the rh-GM-CSF sequence suggests that the carboxy terminus, or D helix, is responsible for the interaction of the cytokine with the β or "shared" subunit. Using hybrid analysis and carboxyl-terminal deletions, Shanafelt et al. (1991b) demonstrated that significant losses of activity were incurred by human (but not mouse) GM-CSF. In particular, residue Trp-122 was acutely sensitive to substitution. Together, these data provide circumstantial evidence for an important role of the carboxyl terminus. In this paper, we provide direct evidence that a specific peptide region in the D, or carboxyl-terminal, helix (110–127) interacts specifically with the α-subunit. It is consistent that the x-ray crystallography of the four-helix bundle structure of h-GM-CSF (Walker et al., 1992; Diederichs et al. 1991) suggests that helix B has very low solvent and helix C has moderate accessibility and that the amino helix A and the carboxyl helix D are solvent-exposed.

Thus, more than one region of rh-GM-CSF is implicated in expression of its varied activities. A role for more than one segment of a ligand in its interaction with receptor is consistent with a model of cytokine action exhibited by human growth hormone. The solution of the structure of the growth hormone co-crystal with its receptor provides information concerning the receptor-ligand binding sites within the complex (deVos et al., 1992). Since this protein is believed to be in the same family as that of rh-GM-CSF, the structure may be a useful model of the complex that rh-GM-CSF forms with its receptor. Interaction of human growth hormone with its homodimer receptor occurs at two distinct regions of the ligand. The first site of interaction with receptor subunit I occurs at the first helix of the ligand. The second site of interaction occurs between the first and fourth helix of the ligand and receptor subunit 2. While the receptor subunits of rh-GM-CSF are not identical, a potentially similar pattern of interaction could be envisioned. Thus, the α receptor subunit of rh-GM-CSF would interact with the helix D or carboxyl terminus of the rh-GM-CSF ligand, whereas the β receptor subunit would interact with the first helix of the ligand as suggested by the studies with murine GM-CSF described by Shanafelt et al. (1992).

The development of the anti-idiotype antibody Ab 1418, which interacts with the α receptor subunit, offers a unique opportunity to probe the heterodimer receptor of rh-GM-CSF. Antibody Ab 1418 provides an invaluable tool for defining the residues on the receptor that may be involved in the ligand-receptor binding site.

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**TABLE II**

| Cell type     | K<sub>H</sub> of high affinity site | K<sub>H</sub> of low affinity site | Inhibition* |
|---------------|-----------------------------------|----------------------------------|-------------|
| AML-193       | 70                                | 100                              |             |
| KG-1<sup>a</sup> | 6.7                               | 0.73                             | 100         |
| JAR           | 2.4                               | 0.74                             | 100         |
| Placental membranes | 0.74                             | 0.74                             | 100         |

*Maximal inhibition of 125I-labeled rh-GM-CSF binding to cell-surface receptors by antibody 1418.

<sup>a</sup> Two murine monoclonal antibodies (Madjic, 1984), one that was raised against KG-1 cells and that recognizes granulocytes (VIM-C6) and one that recognizes a carbohydrate determinant on human granulocytes, monocytes, and their precursors (VIM-2) were tested on KG-1 cells for inhibition of 125I-labeled rh-GM-CSF binding to receptors. The fact that no inhibition was observed with either antibody further supports the specificity of the inhibition derived from the anti-idiotype antibody 1418.
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