Neutralizing and Nonneutralizing Monoclonal Antibodies to the Human Granulocyte-Macrophage Colony-Stimulating Factor Receptor α-Chain

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A panel of monoclonal antibodies was raised against the low-affinity human granulocyte-macrophage colony-stimulating factor (hGM-CSF) receptor α-chain expressed as a recombinant protein on murine FDC-P1 cells. All the selected antibodies were of the IgG2A isotype and bound to protein A. They each recognized both native and recombinant receptors by indirect surface immunofluorescence and by immunoprecipitation. Several of the antibodies also recognized presumably denatured receptors as detected by immunoblotting of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Three different epitopes on the extracellular domain of the GM-CSF receptor α-chain were defined by these antibodies, and two of the epitopes did not appear to be involved in binding hGM-CSF or in interactions with the β-chain of the GM-CSF receptor that are required for high-affinity binding of GM-CSF. On the other hand, the epitope recognized by antibody 287-17-A appeared to be critically involved in the binding of GM-CSF because this antibody completely abrogated both high- and low-affinity binding of GM-CSF to native and recombinant receptors. Antibody 287-17-A had a relatively high affinity for the GM-CSF receptor α-chain (k_0 = 3 nmol/L) and slow dissociation kinetics (k_0 = 0.002 min^-1). These properties made the 287-17-A antibody a potent inhibitor of hGM-CSF biologic action in several different bioassays, with a half-maximal inhibitory dose of about 6 nmol/L (1 μg/mL). This antibody could prove useful in alleviating any pathologic states mediated by excess GM-CSF levels and in defining the domains of the GM-CSF receptor required for ligand binding.

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were harvested for testing. Individual hybridomas were recloned by limiting dilution.

**Immunofluorescence.** COS-7 cells were electroporated with the CD8-expression plasmid encoding the α-chain of the hGM-CSFR or the cell surface molecule intercellular adhesion molecule-1 (ICAM-1). After 3 days, the cells were harvested, washed, and incubated with 50 μL of hybridoma culture supernatant or purified MoAb (10⁶ cells per reaction volume) for 30 minutes at 4°C. The cells were washed, resuspended in fluorescein isothiocyanate derivative of the (Fab³)² fragment of a sheep antimouse Ig antibody (FITC SAMlg-DDAF; Silius, Melbourne, Australia) (1/100) and held on ice for 30 minutes at 4°C. The cells were washed, subjected to fluorescence-activated cell sorting (FACS) or fixed in 1% formaldehyde in phosphate-buffered saline (PBS), and examined by fluorescence microscopy. A similar protocol was used for analyzing supernatants on FADS5 cells.

**Immune depletion.** COS-7 cells electroporated as above were harvested after 72 hours, lysed, and membranes purified as described. The membranes were extracted with 1% (wt/vol) Triton X-100, centrifuged at 100,000g for 15 minutes and the supernatant retained as solubilized hGM-CSFR α-chain. Solubilized hGM-CSFR α-chain (50 μL) was incubated with hybridoma supernatant (100 μL), SAMlg (100 μL of a 1/100 dilution), and protein-G-Sepharose (100 μL of a 1/5 suspension) in a final volume of 400 μL of HEPES-buffered (20 mmol/L, pH 7.4) RPMI-medium containing 10% (vol/vol) FCS (HRF) for 60 minutes at 23°C with rotation. Tubes were then centrifuged at 14,000g for 10 seconds, and the supernatants were recovered. Supernatants were then tested for their content of solubilized hGM-CSFR α-chain using the soluble receptor assay.

**Soluble hGM-CSFR α-chain assay.** Supernatants containing high- and low-affinity binding and biologic responses to GM-CSF.

**MATERIALS AND METHODS**

**Generation of hybridomas.** Murine FDC-P1 cells stably transfected with the hGM-CSFR α-chain cDNA in the CDM8 vector by electroporation (FADA5 cells) were injected intraperitoneally (2 to 3 × 10⁶ cells) into allogenic CBA/J mice. The mice received 3 more weekly injections of the same dose at the same site, and the sera were then assayed in the immunodepletion assay (see below). The mice were then rested for at least 4 weeks before a final immunization. Four days later, the mice were killed by cervical dislocation, and the spleens removed. Single cell suspensions of spleen cells were prepared, washed in serum-free Dulbecco’s modified Eagle’s medium (DME), and fused with NS-1 myeloma cells in the presence of 50% (wt/vol) polyethylene glycol 4000 (Boehringer-Mannheim, Sydney, Australia). The cells were gently resuspended in DME containing 15% (vol/vol) fetal calf serum (FCS) and 10% (vol/vol) of conditioned medium from the P388D1 macrophage cell line (a source of IL-6) and cultured in 96-well Falcon microwell flat-bottom trays (Becton Dickinson, Lincoln Park, NJ) at 2 × 10⁶ cells/well. The cells were selected in HAT medium (hypoxanthine, aminopterin, and thymidine) by overnight culture, and this was repeated every 3 days thereafter. After 7 to 14 days, developing hybridoma clones were observed in the wells, and supernatants were harvested for testing. Individual hybridomas were recloned by limiting dilution.

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**Soluble hGM-CSFR α-chain assay.** Supernatants containing high- and low-affinity binding and biologic responses to GM-CSF.
finity receptors and then incubated with increasing amounts of hGM-CSFR \( \alpha \)-chain (solubilized from transfected COS cells) by co-incubation with different MoAbs or unlabeled hGM-CSF in a solution of 10 pg/mL and hGM-CSF at 5 pg/mL. (Left panel) Ability of the same antibody set to inhibit the binding of \( ^{125} \text{I}-\text{hCM-CSF} \) in the absence of unlabeled GM-CSF minus the nonspecific binding. (Right panel) Inhibition of \( ^{125} \text{I}-\text{hGM-CSF} \) binding to hGM-CSF receptors on COS cells. (B) Inhibition of both high- and low-affinity binding of hGM-CSF to native receptors on HL-60 cells. HL-60 cells were expanded to 2.5 cm of a one-quarter suspension in 0.1 mol/L Na acetate buffer, pH 6.0) for 60 minutes at 23°C with radioiodinated antibody at 4°C (4 hours). Cells were then washed, resuspended in 1 mL HRF containing 50 pg/mL of unlabeled antibody (to prevent rebinding of dissociated radiolabeled antibody). At the indicated times, 50 \( \mu \)L aliquots were removed and bound. Free radioactivity was determined after centrifugation through FCS. The data were plotted as \( \log (\frac{B_r}{B_s}) \) versus time with \( B_r \) being the specific binding at the start of dissociation and \( B_s \) being the specific binding after time \( t \). The kinetic dissociation constant \( (k_d) \) was determined from the half-life of specific binding (\( t_{1/2} \); \( k_d = 0.693/t_{1/2} \) min\(^{-1} \)). The kinetic association rate constant \( (k_a) \) was determined by mixing radiolabeled antibody with cells at 4°C and, at the indicated times, removing aliquots of cells, and separating binding from free antibody as described above. Specific binding was extrapolated to infinite time using a double reciprocal plot to give the amount that would be bound at equilibrium \( (B_e) \). The data were then plotted as \( \log (\frac{B_e}{B_s}) \) versus time to give as the slope, \( k_a \). \( k_a \) was then calculated from the relation \( k_a = k_a \cdot [L] + k_e \cdot [L] \) where \( [L] \) is the initial concentration of the antibody in the mixture. In all cases, specific binding was taken as the difference between binding in the absence and presence of 50 pg/mL of unlabeled antibody. Epitope analysis was performed by titrating the capacity of each unlabeled antibody to compete for the binding of each labeled antibody to hGM-CSFR \( \alpha \)-chains on transfected COS cells.

**Immunoprecipitation.** FDA5 cells (2.5 \( \times \) 10\(^7 \)) were surface iodinated using Na\( ^{125} \text{I} \) (New England Nuclear, North Ryde, New South Wales, Australia) and lactoperoxidase as described, and the cells were lysed in 1% Triton X-100 (Pierce, Rockford, IL) with protease inhibitors as described. Cell lysates were precleared with protein A-Sepharose, and then 25 \( \mu \)L of lystate was incubated with test antibodies at 1 pg/mL (5 \( \mu \)L) for 1 hour at 4°C. Protein A-Sepharose (50 \( \mu \)L) was then added and mixed for 1 hour at 4°C; the beads were washed and extracted with sodium dodecyl sulfate (SDS) sample buffer (50 \( \mu \)L). Samples were applied to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels under reducing conditions and electrophoresed. Gels were dried and exposed to X-ray film for 10 days before development.
**MONOCLONAL ANTIBODIES TO THE hGM-CSF RECEPTOR**

**RESULTS**

To raise MoAbs to the hGM-CSFR α-chain, we first generated a stable transfectant of murine FDC-P1 cells that expressed approximately 10^4 hGM-CSFR α-chain molecules at the cell surface (FDA5 cells). Allogeneic CBA/J mice were immunized with these cells, and mice displaying a positive serum antibody response (as assessed by immunodepletion of solubilized hGM-CSFR α-chain) were used to generate hybridomas by fusion of spleen cells with NS-1 myeloma cells.

Hybridoma supernatants were screened by indirect immunofluorescence on COS cells expressing hGM-CSFR α-chain versus COS cells expressing the ICAM antigen and positive supernatants confirmed by immunodepletion of solubilized hGM-CSFR α-chain. A total of 35 of 390 wells were positive, and the cells in these wells were expanded and cloned by limiting dilution. Of these, 15 hybridoma clones remained positive, and 5 of these were selected for more detailed analysis.

All 5 MoAbs from the hybridoma clones were of the IgG2A isotype and were purified using protein A-Sepharose. Each purified antibody was radiolabeled using either the iodine monochloride or the Bolton-Hunter reagents, and the specific binding to COS cells transfected with the hGM-CSF-α-chain was determined (Fig 1). Specific binding (defined as 100% in Fig 1) was defined as cpm bound in the absence of unlabeled isologous antibody minus cpm bound in the presence of an excess of unlabeled antibody. Antibodies detecting the same or overlapping epitopes on the hGM-CSFR α-chain were shown by their capacity to also inhibit labeled nonisologous antibody binding to these cells when present at the same excess concentration as the isologous antibody. These studies showed that the 2B7-
from FDA5 cells expressing hGM-CSFR α-chain (Fig 2). All antibodies recognized a single major protein of M, approximately 80,000, consistent with the size of hGM-CSFR α-chain, although antibodies 3G9-25-1, 2E9-4-1, and 2C7-16-1, which recognized a common epitope, appeared less efficient than the other two antibodies. A similar size protein was detected by Western blotting of COS-hGM-CSFR α-chain membrane extracts run on SDS gels with antibodies 2B7-17-A, 2C7-16-1, 2E9-4-1, and 3G3-14-15, suggesting that these antibodies might recognize linear epitopes (data not shown).

The 5 antibodies were each tested for their capacity to directly compete for the binding of 125I-hGM-CSF to the hGM-CSFR α-chain solubilized from COS cells. Only antibody 2B7-17-A was able to compete significantly for the hGM-CSF binding site, and, at high concentrations, it competed to the same extent as unlabeled hGM-CSF (Fig 3A). The inability of the other antibodies to compete for the same epitope on the hGM-CSFR α-chain as the 2B7-17-A antibody was again confirmed on transfected COS cells (Fig 3A).

The ability of the 2B7-17-A antibody to inhibit the binding of hGM-CSF to the hGM-CSFR α-chain was dose-dependent, and half-maximal inhibition occurred at approximately 1 μg/mL (6 nmol/L). Reciprocally, hGM-CSF could also inhibit the binding of 125I-2B7-17-A to hGM-CSFR α-chain on COS cells with half-maximal inhibition occurring at a similar dose (approx 5 nmol/L; data not shown). Interestingly, high doses of the 2B7-17-A antibody could also completely inhibit both high- and low-affinity binding of 125I-hGM-CSF to native receptors on HL60 human leukemic cells (that had been induced to differentiate for 5 days with 1.25% dimethylsulfoxide) to the same level as did unlabeled hGM-CSF (Fig 3B).

The binding affinity of the 2B7-17-A antibody for the hGM-CSFR α-chain expressed on COS cells was determined by saturation binding analysis using radioiodinated antibody (Fig 4). Scatchard analysis showed the same number and approximately the same affinity (kD = 3.2 nmol/L) of binding sites on recombinant hGM-CSFR α-chain for the antibody as for hGM-CSF itself. The 2B7-17-A antibody also recognized the native hGM-CSFR α-chain expressed on U937 cells with a similar affinity (kD = 1.4 nmol/L).
We have prepared a panel of murine MoAbs that specifically recognize the low-affinity α-chain of the hGM-CSFR and analyzed five of these in detail. These antibodies define three different epitopes in the extracellular domain of the receptor; two of which do not appear to be involved either in ligand binding or in the interaction of the α-chain with the β-chain of the receptor that is required to generate high-affinity binding and biologic signalling. One antibody recognized an epitope that was essential for ligand binding because this antibody blocked GM-CSF binding and biologic action over the same dose range as required for the primary binding of the antibody to receptor.

All antibodies recognized the hGM-CSFR α-chain that is expressed on a variety of cell types, the receptor extracted from cells in detergent solutions, and a soluble form of the receptor that expresses only the extracellular domain. This suggested that each of the epitopes was expressed in the extracellular domain, that the structure of the extracellular domain was stable in the different forms of the receptor, and that different types of receptor glycosylation did not modify the antibody epitopes. The versatility of these antibodies has been shown by their capacity to detect receptors on intact cells by immunofluorescence and FACS and by binding of radiiodinated antibody. All the antibodies were also able to specifically immunoprecipitate hGM-CSFR α-chain from cell extracts, and most could detect receptor on immunoblots of cell extracts transferred to nitrocellulose from SDS-PAGE gels.

The non-neutralizing antibodies will be useful in enumerating hGM-CSFR α-chains on cells even if the receptors are occupied by endogenous hGM-CSF (eg, from patients undergoing continuous treatment with hGM-CSF). Because they also do not interfere with GM-CSF–induced association of α-chains with β-chains of the receptor or with biologic signalling, they will also be useful in determining, by coimmunoprecipitation experiments in the presence and

DISCUSSION

Because U937 cells express high-affinity receptors for GM-CSF, presumably as a heterodimer of hGM-CSFR α-chains with the common β-subunit, this suggests that the interaction of the α- and β-chains does not interfere with the binding of the 2B7-17-A antibody to its epitope on the α-chain or that αβ complexes do not exist in the absence of hGM-CSF.

The binding kinetics of the 2B7-17-A antibody to the native hGM-CSFR α-chain expressed on U937 cells was also investigated at 4°C (Fig 5). Both association and dissociation kinetics were consistent with a simple bimolecular interaction with an association rate constant of 3.1 × 10⁶ (mol/L)⁻¹ min⁻¹, and a dissociation rate constant of 0.002 min⁻¹. The ratio of these constants yields a calculated kd of 0.7 nmol/L in reasonable agreement with the values measured at equilibrium at 4°C (kd = 1 to 3 nmol/L).

The slow kinetic dissociation rate and relatively high affinity of the 2B7-17-A antibody for hGM-CSFR α-chain as well as its capacity to inhibit the binding of hGM-CSF to both low- and high-affinity GM-CSFRs suggested that it could act as a very effective inhibitor of hGM-CSF biologic action. This was confirmed in three separate biologic assays.

FDA5 cells (FDC-P1 cells transfected with hGM-CSFR α-chain) show only low-affinity binding of hGM-CSF, and, although they have become partially autonomous for growth, colony numbers in semisolid agar were augmented by hGM-CSF in the range expected for low-affinity binding (10¹ to 10² U/mL or 0.1 to 50 nmol/L). At high doses, the 2B7-17-A but not the isotype-matched 3G3-14-15 antibody suppressed colony formation induced by hGM-CSF by more than 80%, and the dose of antibody required for 50% inhibition of hGM-CSF–induced colonies was about 1 µg/mL (Fig 6). This corresponds to an antibody concentration of about 6 nmol/L and is consistent with the primary antibody binding affinity to hGM-CSFR α-chain.

AML-193 cells (a leukemic human cell line) display both high- and low-affinity hGM-CSFRs, and their proliferation is stimulated by hGM-CSF. Again antibody 2B7-17-A but not 3G9-25-1 was able to reduce hGM-CSF–stimulated thymidine incorporation into AML-193 cells to background levels and half-maximal inhibition was observed at about 0.5 µg/mL (3 nmol/L; Fig 7).

Finally, antibody 2B7-17-A but not 3G3-14-15 was able to inhibit both day-7 and day-14 colony formation in agar by normal human bone marrow cells displaying native high-affinity hGM-CSFRs. In this case, half-maximal colony formation was induced by 50 U/mL (35 pmol/L) of hGM-CSF, and the 2B7-17-A antibody inhibited colony formation at a half-maximal dose of about 1 µg/mL (6 nmol/L, Fig 8).
absence of GM-CSF, those molecules that become associated with the GM-CSFR after ligand binding, which may be important in signal transduction.

In contrast, the neutralizing antibody (2B7-17-A) probably recognizes the primary ligand-binding site on the hGM-CSFR α-chain. The inhibitory capacity of this antibody in all assays correlated well with its primary binding affinity and suggested a direct two-way competition of the antibody and GM-CSF for a common binding site rather than steric hindrance. Therefore, molecular definition of this binding epitope should help in defining the interaction surface on the receptor for GM-CSF. This can be achieved by analysis of the ability of synthetic peptides to bind to the antibody and by analysis of proteolytic fragments of the hGM-CSFR α-chain by immunoblotting. This approach might succeed because the ability of antibody 2B7-17-A to recognize SDS-denatured receptor on immunoblots suggests that it may recognize a linear peptide epitope.

Antibody 2B7-17-A inhibited low- and high-affinity binding and biologic action of hGM-CSF at the same doses defined by its primary-binding affinity to the isolated hGM-CSFR α-chain. The two current models for formation of high-affinity signal-competent GM-CSFR complexes are (1) that GM-CSF binds to the α-chain and then induces an interaction with the β-chain or (2) that GM-CSF binding stabilizes pre-existing α-β chain complexes. Our data with antibody 2B7-17-A, although it cannot distinguish between these two models, imply that, if the second model is correct, then the association of α- and β-chains does not in any way block access of even a bulky antibody molecule to the ligand-binding site. This seems unlikely if the GM-CSF molecule is proposed to directly contact the β-chain as might be expected by analogy with the homologous growth hormone-receptor-complex X-ray structure.  

It is hoped that these MoAbs might be useful in the treatment of diseases that are thought to result, at least in part, from either the excess or inappropriate production of GM-CSF or its receptor. These MoAbs may also be used in targeting for destruction cells that express the GM-CSFR (eg, leukemic cells or cells mediating autoimmunity), and they should also be useful in defining the molecular pathways by which GM-CSF induces biologic signals.

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