Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a member of the four-helix bundle family of cytokines/growth factors which exhibit several activities. It is a hematopoietic growth factor, a cytokine involved in inflammatory and immune processes, an adjunct for cancer therapy, and an anti-tumor immunomodulator. Studies of interactions between GM-CSF and its receptor and identification of small peptides presenting binding capacity to the receptor are important goals for the development of GM-CSF analogs. Here we describe the study of two cyclic peptides, 1785 and 1786, developed based on structural analysis of the GM-CSF region mimicked by anti-GM-CSF recombinant antibody 23.2. These peptides were designed to structurally mimic the positions of specific residues on the B and C helices in human GM-CSF implicated in receptor binding and bioactivity. Both 1785 and 1786 were specifically recognized by polyclonal anti-GM-CSF antibody (stronger for 1786 than 1785). 1786 also competitively inhibited binding of GM-CSF to the GM-CSF receptor on HL-60 cells and demonstrated antagonist bioactivity, as shown by its reversal of GM-CSF’s ability to inhibit apoptosis of the GM-CSF-dependent cell line M07E. These studies support the role of residues on the GM-CSF B and C helices in receptor binding and bioactivity and suggest strategies for mimicking binding sites on four-helix bundle proteins with cyclic peptides.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a hematopoietic growth factor and a cytokine involved in many inflammatory and immune processes. GM-CSF activates antigen-presenting cells (monocytes, macrophages, and dendritic cells), increases major histocompatibility complex class II expression-enhancing antigen presentation, and increases macrophage anti-tumor activity (1). Recently it has been used as an important adjunct in cancer therapy for bone marrow recovery following chemotherapy and transplantation (2). Moreover, GM-CSF induces protective immune responses against lymphoma cells if fused with a tumor-derived idiotype, eliciting tumor-specific immunity (3). GM-CSF also enhances the immunogenicity of tumor cells when expressed by them, resulting in induction of protective anti-tumor immunity, while other cytokines such as IL-2, IL-4, IL-5, IL-6, γ-interferon, or tumor necrosis factor-α are less effective (4).

The crystal structure of human GM-CSF (5–8) reveals a four-helix bundle organization similar in some respects to that described for growth hormone (9), IL-2 (10), and IL-4 (11–14). The related cytokines macrophage colony stimulating factor (15) and IL-5 are organized as dimers of four-helix bundles (16). GM-CSF activity is mediated by binding to specific cellular receptors (GM-CSFR) which belong to a recently described supergene family (17–23). The high affinity GM-CSFR is comprised of an α chain (GM-CSFRα) specific for GM-CSF (20), and a β chain (βc), which can also associate with the IL-3 and IL-5 receptor α chains (21). The GM-CSFRα imparts specificity to the interaction with GM-CSF, and when expressed without βc is able to bind GM-CSF, albeit with lower affinity than the heterodimeric receptor (24). The high affinity receptor (GM-CSFRα and βc) appears to be the signal-transducing unit (25, 26), with a sequential binding of GM-CSF to GM-CSFRα followed by binding to βc postulated.

GM-CSF and the related four-helix bundle cytokines are important targets for drug design and production of low molecular weight analogs which mimic the native ligand. Studies of ligand-receptor intermolecular interactions which help delineate their active sites should allow the development of small molecules able to mimic the larger polypeptide ligands. Such small drugs, created based on analysis of the most important binding interactions, could circumvent problems of immunogenicity, antigenicity, rapid proteolysis by serum proteolytic enzymes, short serum half-life, and low oral bioavailability, commonly presented by large polypeptides.

In prior studies, linear peptide analogs of GM-CSF were produced by dividing the human GM-CSF sequence into six peptides (27). This strategy led to the identification of two peptides with receptor binding and antagonist activity. One peptide corresponding to residues 17–31 (the A helix) inhibited high affinity receptor binding, while a second peptide corresponding to residues 54–78 (the B and C helices) inhibited low affinity receptor binding (27). This implicates these sites in intermolecular interactions with the GM-CSFR. We also have used a recombinant antibody (rAb) as a GM-CSF mimic (28). Molecular modeling of the rAb 23.2 allowed the identification of complementarity determining regions (CDRs) as sites of structural mimicry of GM-CSF, focusing attention on the CDR1

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* This work was supported in part by grants from the American Cancer Society and the Arthritis Foundation (to W. V. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a grant from the USAMRAA (DA08-1794-4310) Breast Cancer Initiative.

‡ Supported by grants from the American Foundation for AIDS Research and the National Institutes of Health.

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1 The abbreviations used are: CSF, colony-stimulating factor; GM, granulocyte-macrophage; CSFR, colony-stimulating factor receptor; IL, interleukin; rAb, recombinant antibody; TPA, 12-O-tetradecanoylphorbol-13-acetate; CDR, complementarity determining region; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.
region mimicking residues on the B and C helices of GM-CSF. After synthesis and characterization of CDRI, CDRII, and CDRII peptides, the CDRI peptide exhibited specific GM-CSF receptor binding and antagonist bioactivity (28). Thus, these studies suggest that residues on the B and C helices of GM-CSF mediate binding to the low affinity receptor (GM-CSFRα alone).

Here we describe the development of two cyclic peptide GM-CSF mimics (1785 and 1786) obtained from structural analysis of the GM-CSF region mimicked by rAb 23.2 (28). Cysteines were introduced in the peptide structures at the amino and carboxyl termini to allow cyclization. The cydized peptides were specifically bound by polyclonal anti-GM-CSF antibody (stronger for 1786 than for 1785). Moreover, 1786 competes with GM-CSF for binding to the GM-CSF receptor present on HL-60 cells and reverses GM-CSF’s prevention of apoptosis of MO7E cells. Thus, 1786 represents a structurally designed biological and receptor antagonist of GM-CSF.

**MATERIALS AND METHODS**

Design of Peptides 1785 and 1786—The formation of peptide intrachain disulfide bridges was determined by solubility phase methods, deprotected, and released from the resin by anhydrous HF as described previously (29–32) by Macromolecular Resources at Colorado State University (Fort Collins, CO). Peptide 1785 was designed beginning at Thr-57 and proceeding up the exposed residues on the B helix (GLU-60 and LYS-63), then continuing in the reverse orientation on the C helix (LYS-74, THR-78, SER-82, and LYS-85). Based on the ability of a predicted reverse turn structure (the 23.2 CDRI) to functionally mimic its individual CDR sequences with the human GM-CSF structure (28). Important residues in the GM-CSF structure mimicked by similar residues on 23.2 were postulated as Thr-57, Glu-60, Lys-63, Lys-74, Thr-78, Ser-82, and Lys-85. Glycine or alanine residues were introduced to orient the predicted contact residues on the same face of the reverse turn. Additional Gly residues were added at the amino and carboxyl termini to appropriately position Cys residues for cyclization by disulfide bridge formation. The 1785 peptide was designed according to the same principles, but beginning with Lys-85 on the C helix and proceeding in the opposite orientation. The sequences of these peptides and their predicted structures in comparison with the GM-CSF structure is shown in Fig. 1.

Preparation of Cyclic Peptides—The two peptides were synthesized by solid phase methods, deprotected, and released from the resin by anhydrous HF as described previously (29–32) by Macromolecular Resources at Colorado State University (Fort Collins, CO). Peptide 1785 was designed beginning at Thr-57 and proceeding up the exposed residues on the B helix (GLU-60 and LYS-63), then continuing in the reverse orientation on the C helix (LYS-74, THR-78, SER-82, and LYS-85). Based on the ability of a predicted reverse turn structure (the 23.2 CDRI) to functionally mimic its individual CDR sequences with the human GM-CSF structure (28). Important residues in the GM-CSF structure mimicked by similar residues on 23.2 were postulated as Thr-57, Glu-60, Lys-63, Lys-74, Thr-78, Ser-82, and Lys-85. Glycine or alanine residues were introduced to orient the predicted contact residues on the same face of the reverse turn. Additional Gly residues were added at the amino and carboxyl termini to appropriately position Cys residues for cyclization by disulfide bridge formation. The 1785 peptide was designed according to the same principles, but beginning with Lys-85 on the C helix and proceeding in the opposite orientation. The sequences of these peptides and their predicted structures in comparison with the GM-CSF structure is shown in Fig. 1.

Peptide Characterization—The preparation of peptide intrachain disulfide bridge versus interchain bridges was estimated by mass spectrometry analysis performed at the Protein Chemistry Laboratory of the University of Pennsylvania School of Medicine (J. Lambris). This indicated ~90% monomers of the oxidized peptides.

**Enzyme-linked Immunosorbent Assay (ELISA)**—ELISA was performed with polysynthetic plates (Dynatech Laboratories Inc., Chantilly, VA). The peptides 1785, 1786, and a control peptide (Cys-Thr-Tyr-Arg-Tyr-Pro-Leu-Glu-Leu-Asp-Thr-Ala-Asn-Asn-Arg) were dissolved in 50 mM NH4HCO3 at 120, 90, 60, and 30 μM and 50 μM of each dilution were used to coat the wells in duplicate by evaporation overnight at 37°C. As positive controls wells were coated with 50 μM of 1 μg/ml GM-CSF in 0.1 M NH4HCO3, pH 8.0, overnight exposed to the air at room temperature. The extent of oxidation of peptide-coated wells (34) was determined from the absorbance at 420 nm, using the formula: (100% sulfhydryls was determined from the absorbance at 450 nm was detected using the plate reader MR 5000 (Dynatech Laboratories Inc., Chantilly, VA). Values were reported subtracting the absorbance measured for uncoated wells from the absorbance of peptide-coated wells (34).

Radioreceptor Binding Assay—Binding of 1785 and 1786 to the GM-CSF receptor present on HL60 cells was analyzed by a competitive radioreceptor assay modified from previously reported protocols (20, 35). Briefly, HL60 (from ATCC) were grown in RPMI 1640 with 10% fetal calf serum, l-glutamine, oxalate, pyruvate, insulin, essential amino acids, and nonessential amino acids. 106 cells were washed twice in RPMI 1640, 10 mM Hepes, pH 7.4, 10% fetal calf serum (binding buffer), centrifuged, and incubated with different dilutions of peptides 1785, 1786, and control peptide (for final concentrations of 500, 250, 125, 62.5, and 31.25 μM) for 1 h at room temperature. 125I-GM-CSF (0.5 nM), and cold GM-CSF (at the saturating concentration of 50 nM) were added for binding and incubated at room temperature for 1 h. The mixture was then layered over 500 μl of chilled fetal calf serum and centrifuged, and the counts/min bound determined in an LKB gamma counter. Specific binding was determined by subtracting the nonspecific counts/min bound from the total counts/min bound. Scatchard analysis revealed that, at this concentration, predominately low affinity sites (2.9 nM) were measured (27) (data not shown). Based on the EC50 achieved by peptide, the K, was calculated by the method of Cheng and Prusoff (36).

Inhibition of Apoptosis—The assay was performed in a 24-well polystyrene plate (Corning, Costar Corp., Cambridge, MA), using MO7E cells (from R. Zolner, Genetics Institute, Cambridge MA), grown in RPMI 1640, 10% fetal calf serum, l-glutamine, oxalate, pyruvate, insulin, nonessential amino acids, essential amino acids, penicillin/streptomycin, and 20% U87 supernatant (containing GM-CSF as a growth factor). The peptides were added at different dilutions to the wells (final peptide concentrations of 160, 80, 40, 20, 10, 5, and 0 μM). After peptide treatment, the plates under UV light for 40 min, fixed amounts of sterile GM-CSF (200 pmol), TPA (12-O-tetracosanoylphorbol-13-acetate (Sigma), 4 nm), and U87 supernatant (5%) were added separately to all the different concentrations of peptide. 250 μl of cell suspension at 106 cells/ml, previously washed with the medium without U87 supernatant and resuspended in the same medium, were added to each well, reaching a final concentration 5 × 104 cells/ml in a total volume of 500 μl. After 24 h of growth, cells were lysed and DNA degradation detected both by an agarose gel run and by the use of “Cell Death Detection ELISA” kit (Boehringer Mannheim).

For the agarose evaluation, 350 μl of 5 × 105 cells/ml were washed, added to 20 μl of lysis buffer (10 mM EDTA, 50 mM Tris HCl pH 8.0, 0.5% Naoctysarcosine, sodium salt (Sarkosyl), 0.5 mg/ml proteinase K) and incubated for 1 h at 50°C. After addition of 10 μl of 0.4% RNase and incubation at 50°C for 1 h, the samples were mixed with 10 μl of 10 mM EDTA, PH 8.0, 0.03% bromphenol blue, 1% Nue Seeve GTG agarose (FMC BioProducts, Rockland, ME), heated at 70°C for 10 min, loaded into a 1.2% agarose gel and run for 1 h at 100 V using TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA pH 8.0). The gel was stained with ethidium bromide (Sigma) and photographed under ultraviolet light.

Mono- and oligonucleoside fragments present in the cytoplasmic fraction of cell lysates were detected following the protocol for “Cell Death Detection ELISA” kit. Briefly, the microtiter plate was coated with 0.1 M tris(hydroxymethyl)aminomethane solution of the lyase derived from 2.5 × 106 cells, DNA was detected by the anti-DNA-peroxidase system according to the kit instructions, with color development read at 405 nm.

**RESULTS**

Peptide Design—The two peptides were synthesized according to the sequences reported in Fig. 1. The postulated contact
residues on the GM-CSF B and C helices (regions involved in the interaction with GM-CSFR) were introduced into the peptides in two different orientations ("up" the B and "down" the C helices for 1786, with the opposite orientation for 1785). The design incorporated reverse turn structures together with appropriate spacer residues and cysteines at the amino and carboxyl termini, which allowed the development of cyclic forms.

**Peptide Cyclization**—The procedure followed for peptide cyclization was oxidation of the terminal sulfhydryls with intrachain disulfide bond formation. Ellman determination indicated that only 4.7 and 1.8% of free sulfhydryls were still present in the oxidized forms of 1785 and 1786, respectively, confirming near complete oxidation of the peptides.

Mass spectrometry analysis was performed on the oxidized peptides to confirm that oxidation had resulted in intrachain disulfide bond formation, as opposed to formation of oligomers. The mass spectrometry study showed that >90% of the oxidized 1785 peptide was represented by a peak at molecular mass 1514, with the theoretical molecular mass for 1785 being 1511 daltons. Similarly, >90% of the 1786 peptide was seen as a peak at molecular mass 1637, the theoretical molecular mass being 1639. Thus, both of the oxidized peptides were >90% in the monomeric form, with only trace contamination by oligomers (dimers and trimers).

**Recognition of Peptides by Polyclonal Antibody against GM-CSF**—The ability of these peptides to mimic GM-CSF was initially evaluated by its recognition by polyclonal antibody against GM-CSF in an ELISA (Fig. 2). Neither peptide showed any specific binding by the preimmune serum (normal mouse serum), indicating lack of nonspecific binding. Both peptides 1785 and 1786 were specifically bound by polyclonal antibody against GM-CSF, with the titer higher for 1786 than that for 1785. The control peptide was not bound by the anti-GM-CSF, further supporting specific recognition of the peptide mimics. This supports structural mimicry of GM-CSF by the peptides 1785 and 1786.

**Peptide Binding to the GM-CSF Receptor**—The ability of the peptides to bind the GM-CSF receptor was evaluated by their ability to compete with GM-CSF for binding to the GM-CSF receptor present on HL-60 cells as evaluated by a radioreceptor assay. Peptides were preincubated with HL-60 cells prior to the addition of 125I-GM-CSF, and specific binding was determined by carrying out identical reactions in the presence of excess of unlabeled GM-CSF. Fig. 3 reports the typical results obtained with 1786, 1785, and control peptides. While 1785 and control peptides failed to show any specific inhibitory activity, 1786 inhibited GM-CSF binding to its receptor in a dose-dependent manner, with 50% inhibition achieved at 0.500 μg/ml. 1786 therefore antagonizes GM-CSF binding to its receptor, indicating binding of this peptide to the GM-CSFR on HL-60 cells. Scatchard analysis of GM-CSF binding to HL-60 cells reveals high affinity (46 pM) and low affinity (2.9 nM) sites for GM-CSF binding (20, 35). Under the conditions of the assays here, predominantly low affinity sites were measured (27) (data not shown). Based on the low affinity K_d, calculation of the K_i for peptide using the method of Cheng and Prusoff (36) gives a value of 270 μM.

**Bioactivity of Peptides**—GM-CSF bioactivity can be evaluated by its ability to inhibit spontaneous apoptosis of the GM-CSF-dependent cell line MO7E (37, 38). This assay is of particular utility as it can also be applied to stimuli which inhibit apoptosis independent of signaling through the GM-CSF receptor (37). To analyze the bioactivity shown by the 1786 and 1785 peptides, their capacity to interfere with GM-CSF's ability to prevent apoptosis in MO7E cells was assayed. Apoptosis was evaluated both by agarose gel electrophoresis of total cellular DNA and by a specific ELISA assay. In addition to GM-CSF, two other stimuli were evaluated: phorbol ester (TPA), which inhibits apoptosis in a receptor-independent fashion, and GM-CSF containing U87 cell supernatant.

Both the agarose gel and the ELISA results (Fig. 4) indicated clear antagonist activity for the 1786 peptide, with reversal of...
GM-CSF's prevention of apoptosis. Increasing the amount of presence of GM-CSF increased the amount of apoptosis seen (IC50 of 85 μM). When incubated with the cells in medium alone, the 1786 peptide did not prevent DNA degradation, excluding any agonist activity by the peptide. The same peptide, in the presence of U87 cell supernatant, presented the same type of dose-dependent behavior in increasing apoptosis as shown in presence of GM-CSF (IC50 of ~65 μM). The 1786 effect was not seen in the presence of TPA which prevents apoptosis in a receptor independent fashion, indicating that the antagonist activity was GM-CSF receptor-dependent. In contrast, the 1785 peptide did not demonstrate agonist or antagonist activity in these apoptosis assays. This indicates that 1786, which inhibits GM-CSF receptor binding, has a similarly specific GM-CSF receptor-dependent antagonist bioactivity.

DISCUSSION

The interaction of GM-CSF with its receptor has been the subject of intense investigation. Prior studies with GM-CSF mutants indicated that residues on the first (A) helix of GM-CSF are involved in the binding to high affinity receptor (the GM-CSFRαβc complex) but not to low affinity receptor (GM-CSFRα alone) (24, 39, 40). This is illustrated most strikingly by studies using mutants of residue Glu-21 of GM-CSF, which inhibit binding of GM-CSF to the low affinity receptor, but display little activity in inhibiting binding to the high affinity receptor (39, 41, 42). Based on these experiments, it has been proposed that the first α helix of GM-CSF is responsible for binding to βc (40).

Murine and human GM-CSF display species specificity and are not cross-reactive. As substitutions are scattered throughout these molecules, it was possible to swap regions of murine and human GM-CSF to locate sites critical for receptor interaction (35). These studies indicated a critical role for amino acids 21–31 (A helix) and 77–94 (including the C helix) in mediating the activity of human GM-CSF, suggesting that the second site may be involved in binding to the GM-CSFRα. Additional mutagenesis studies (42–45), mapping of neutralizing monoclonal antibodies (46–50), and synthetic peptide studies (47, 51, 52) suggest other potential interaction sites. Thus, in spite of considerable study, the GM-CSFRα interaction site(s) on GM-CSF remain incompletely characterized.

In our group use of synthetic peptides, anti-peptide antisera, and neutralizing monoclonal antibody to map epitopes on GM-CSF important for bioactivity have led to several conclusions: a peptide corresponding to residues 17–31 of the A helix, as well as antibodies against this peptide, are able to inhibit GM-CSF dependent cellular proliferation; the 17–31 peptide also inhibits GM-CSF binding to the high affinity receptor but not to the
low affinity receptor; a peptide corresponding to residues 54–78 overlapping the B and C helices is recognized by two neutralizing monoclonal antibodies to GM-CSF and exhibits antagonist bioactivity (27). This suggests a model of receptor interaction where residues on the B and C helices of GM-CSF, the opposite face of the A helix, are involved in interactions with GM-CSF receptors, while residues on the A helix mediate binding to β2 (27). This model is supported by analysis of a mAb mimic of GM-CSF (23.2) as well as a peptide derived from the CDRI sequence of the mAb 23.2. The CDRI peptide and the mAb were shown to exhibit structural similarity to residues on the GM-CSF B and C helices; both the peptide and the mAb mimic were bound by neutralizing anti-GM-CSF monoclonal antibody 126.213 and exhibited biological and/or receptor antagonist activity (28, 33).

The purpose of this study was to further test this model by developing additional peptides which mimic the position of specific residues on the GM-CSF B and C helices, and evaluating them for receptor binding and biological activity. The structure of the two peptides discussed in this report derived from our prior studies, with 1785 and 1786 designed to structurally mimic potential contact residues on the GM-CSF B (residues 54–61) and C (residues 77–83) helices. The two peptides were synthesized with cysteines at both the amino and carboxyl termini in order to develop cyclic forms, thereby constraining the conformations of the peptides and providing more accurate mimicry of the B and C helical face of GM-CSF. The cyclic peptides also allowed us to evaluate whether reverse turn peptide mimics, such as those developed from the mAb 23.2 CDRI sequence, could be developed from simple structural considerations, obviating the need to develop them by library screening or antibody mimicry.

The cyclic peptides were easily prepared by oxidation overnight, reaching almost 100% oxidation and 90% yield (only traces of dimer and trimer were detected by mass spectrometry analysis). The cyclized monomer peptides were therefore used in binding tests to polyclonal antibody against GM-CSF and to GM-CSF receptor present on HL60 cells. In both cases peptide 1786 showed good binding capacity, displaying competitive behavior toward GM-CSF in the radioreceptor assay. On the other hand, peptide 1785 demonstrated a lower binding affinity to polyclonal anti-GM-CSF antiserum and complete lack of interaction with the GM-CSF receptor. In order to establish their bioactivity, the peptides were assayed in an apoptosis assay. GM-CSF is known to prevent apoptosis of MO7E cells (37, 38). These cells were incubated, in presence or absence of different concentrations of peptides, with fixed amounts of GM-CSF or U87 supernatant (a source of GM-CSF). TPA, an agent which also prevents apoptosis but via a different mechanism not involving the GM-CSF receptor, allowed the specificity of the reaction to be evaluated (37). Peptide 1786, but not 1785 or control peptides, displayed biological antagonist activity: increasing the amount of peptide 1786 resulted in an increase in apoptosis in response to GM-CSF or U87 supernatant, while no effect was seen in the presence of TPA.

The IC50 for peptide 1786 in the apoptosis assay was similar for both GM-CSF and U87 supernatant (65–85 μM). This is somewhat smaller than the calculated Kᵢ for peptide inhibition of binding to low affinity receptor sites on HL-60 cells (270 μM). However, the low affinity sites do not appear to mediate bioactivity, while the high affinity sites do (21, 39). Interestingly, if a similar EC50 is assumed for the high affinity sites, the calculated Kᵢ for peptide 1786 in the binding assay is 59 μM (36), much closer to the IC50 observed in the apoptosis assay. This supports the role of high affinity sites in mediating bioactivity.

Based on these studies, peptide 1786 represents a receptor antagonist of GM-CSF, supporting our conclusions from molecular-structural analysis utilizing recombinant antibodies (28) for the identification of residues critical for bioactivity. Moreover, these studies suggest that similar peptide mimics can be designed based on structural information derived from knowledge of potential contact residues. The ability to design such mimics may be readily extended to other systems where sufficient structural and biological information is available to delineate potential contact residues. This should allow for the analysis of potential contact residues on novel backbones as
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well as the rational design of receptor antagonists with potential clinical utility.

Acknowledgments—We thank the Protein Chemistry Laboratory of the Medical School of the University of Pennsylvania for the mass spectrometry analysis, Paul McGonigle and Carl Romano for their help with pharmacology questions, and A. Domenico for his helpful comments.

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