Site-Specific Conjugation of Diethylenetriaminepentaacetic Acid to Recombinant Human Granulocyte-Colony-Stimulating Factor: Preservation of Protein Structure and Function

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ABSTRACT: The chelating agent diethylenetriaminepentaacetic acid (DTPA) was conjugated site-specifically to the N-terminus of recombinant human granulocyte-colony-stimulating factor (rhG-CSF) by reaction of the protein with DTPA dianhydride at an initial pH of 6.0. The reaction was efficient in that 84% of the starting rhG-CSF was N-terminally modified and could be purified to homogeneity by cation-exchange chromatography. Chelation of ^{111}In by the DTPA−rhG-CSF conjugate was demonstrated by cation-exchange HPLC and thin-layer chromatography. Metal contamination of conjugate preparations, as well as metal-loading onto the conjugate, could be monitored by either cation-exchange HPLC or isoelectric focusing. The 1:1 stoichiometric molar ratio of DTPA to protein for the DTPA−rhG-CSF conjugate was determined by thin-layer chromatography and mass spectrometry, and the localization of the conjugated DTPA moiety was resolved using a peptide mapping procedure. The secondary structure (i.e., α-helicity) of the protein was unmodified following conjugation as revealed by circular dichroism. Furthermore, the conjugate induced a similar induction of peripheral WBC counts as unmodified rhG-CSF when injected subcutaneously into hamsters, demonstrating preservation of protein bioactivity. These results reveal a simple and efficient method for conjugating DTPA to protein, via reaction with the dianhydride, to yield a homogeneous and well-defined product. The procedure may prove to be a useful method of labeling growth factors and related proteins while preserving structural and functional integrity.

Covalent coupling of diethylenetriaminepentaacetic acid (DTPA)[1] to proteins using DTPA dianhydride has been described by several investigators (Hnatowich et al., 1983a; Lauffer & Brady, 1985; Ogan et al., 1987). The protein-bound chelate can form complexes with a variety of metallic radionuclides (Meares & Goodwin, 1984), paramagnetic metal ions (Lauffer & Brady, 1985; Ogan et al., 1987), and fluorescent metals (Mukkala et al, 1989). The conjugation is routinely performed at pH ≥7.0 (Hnatowich et al., 1983a), where the dianhydride reacts primarily with free amine groups to form amide bonds. The number of DTPA groups conjugated to the protein is often given as an average number, as sample preparations are heterogeneous, each having protein with both more and less chelating groups than the average number (Hnatowich & McGann 1987). Because the reactive DTPA molecule is a dianhydride, there exists the undesirable potential for protein−protein cross-linking (Hnatowich et al., 1983b). For example, reaction of DTPA dianhydride with insulin yielded a complex mixture of several products, including cross-linked protein and acylated tyrosine residues (Maisano et al., 1992).

Granulocyte-colony-stimulating factor (G-CSF) is a glycoprotein which induces differentiation of hematopoietic precursor cells to neutrophils, and stimulates the activity of mature neutrophils (Layton, 1992). G-CSF is a member of a class of cytokines sharing a common structural motif of a four α-helix bundle with two long crossover connections (Hill et al., 1993). This family includes granulocyte/macrophage-colony-stimulating factor (Diederichs et al., 1991), growth hormone (De Vos et al., 1992), interferon β (Senda et al., 1992), interleukin-2 (IL-2) (Bazan, 1992), and interleukin-4 (IL-4) (Powers et al., 1992). Recombinant human G-CSF (rhG-CSF), expressed in *Escherichia coli*, contains 175 residues, has a molecular mass of 18 798 Da, and is biologically active (Souza et al., 1986).

By initiating the reaction of rhG-CSF with DTPA dianhydride at a relatively low initial pH of 6.0, we demonstrate specific conjugation of the chelating group to the N-terminus. The well-defined monomeric protein conjugate can be highly purified following the one-step reaction. The protein retains full secondary structure and bioactivity following the conjugation. Chelation of indium induced a slight decrease in secondary structure, but did not affect the observed bioac-

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[1] Abbreviations: ABD-F, 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; AML, acute myeloblastic leukemia; CD, circular dichroism; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; EndoLys-C, Endoproteinase Lys-C; FPLC, fast protein liquid chromatography; G-CSF, granulocyte-colony-stimulating factor; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing; IL-2, interleukin-2; IL-2R, IL-2 receptor; IL-4, interleukin-4; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; PAGE, polyacrylamide gel electrophoresis; rhG-CSF, recombinant human G-CSF; SDS, sodium dodecyl sulfate; TBP, tributylphosphine; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; WBC, white blood cell.

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tivity. This procedure provides an alternative to the relatively difficult procedure of iodinating G-CSF (Nicola & Metcalf, 1984; Avalos et al., 1990), and may prove useful for labeling other growth factors and related proteins.

EXPERIMENTAL PROCEDURES

Materials. Recombinant human G-CSF (rhG-CSF) was prepared as described (Souza et al., 1986). Diethylenetriaminepentaacetic acid (DTPA) dianhydride and tributylphosphine (TBP, technical grade) were purchased from Aldrich (Milwaukee, WI). Trifluoroacetic acid (TFA) was obtained from Pierce Chemicals (Rockford, IL). Endoproteinase Lys-C (EndoLys-C) and 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) were obtained from Wako Chemicals (Richmond, VA). $^{111}InCl_3$ (carrier-free) was from New England Nuclear (Boston, MA). α-Cyano-4-hydroxycinnamic acid was purchased from Biomolecular Separations Inc. (Reno, NV). HPLC-grade water was obtained using a Millipore Milli-Q water system (Milliford, MA) operating at 18 MΩ. All other reagents were of analytical grade.

Conjugation Procedure. DTPA dianhydride was placed in a dry acid-washed (Meares et al., 1984) test tube. One to two milliliters of anhydrous chloroform was then added, and the tube was vortexed under a light stream of nitrogen gas to evaporate the chloroform and form a thin film of the DTPA dianhydride on the walls of the tube. Unless stated otherwise, rhG-CSF at a concentration of 2.75−4.00 mg/mL in 100 mM sodium phosphate buffer, pH 6.0, was added to the DTPA dianhydride-coated tube (DTPA dianhydride:rhG-CSF ratio, 50:1, mol/mol) while gently swirling. Unconjugated DTPA was removed by passage through a G50 spin column as described (Penefske, 1979). Alternatively, DTPA-conjugated protein was isolated by preparative cation-exchange chromatography as described below.

SDS−Polyacrylamide Gel Electrophoresis. SDS−PAGE was performed using 17−27% ISS MiniPlus gels (Nattick, MA). Samples were diluted with nonreducing buffer, and 5 μg of protein was loaded into each well. The gels were run on a discontinuous buffer system and stained with Coomassie Blue R-250 (Laemmli, 1970).

Size-Exclusion HPLC. HPLC was performed on a Waters Liquid Chromatograph (Milliford, MA) equipped with a WISP 717plus autosampler refrigerated at 5 °C, and a 490E multiwavelength UV/Vis detector in line with a Raytest Ramona LS radioisotope detector (Pittsburgh, PA). The void volume between the UV/Vis detector and radioisotope detector was 50 μL.

For size-exclusion HPLC, samples were analyzed with an isocratic mobile phase of 0.1 M sodium phosphate buffer, 0.5 M NaCl, pH 6.9, on a Phenomenex BioSep S2000 column (Torrance, CA) eluted at 1.0 mL/min at 25 °C. Elution was monitored for absorbance at 280 nm and recorded by Waters Millennium software on a PC computer.

Ion-Exchange HPLC. Analytical and preparative cation-exchange chromatography were performed with mobile phases of buffer A (20 mM sodium acetate) and buffer B (20 mM sodium acetate, 0.5 M NaCl); both were at pH 5.4.

Analytical samples were injected with the Waters HPLC system onto a TosoHaas SP-5PW, 7.5 × 75 mm column (Montgomery, PA) equilibrated with mobile phase A. Separation was performed at 25 °C with a 1% B/min linear gradient over 30 min at 1.0 mL/min. Separation was detected by monitoring absorbance at 220 nm and, when applicable, with the radioisotope detector. For samples containing indium, 1 mM EDTA was added to buffer A before adjustment of the pH.

Samples for preparative cation-exchange chromatography were diluted to 50 mL with Milli-Q water to lower the ionic strength below that of buffer A, thus allowing adsorption onto a Pharmacia Hi-Load SP-Sepharose High Performance, 16/10, strong cation-exchange column (Pharmacia, Sweden). Separation was accomplished at 5 °C by a FPLC system equipped with a 50 mL injection loop from Pharmacia. Elution was carried out with a 0−40% buffer B gradient over 180 min at 1.0 mL/min. Elution was monitored for absorbance at 280 nm and recorded with a PC computer using FPLC Director software from Pharmacia. Pooled fractions from the SP-Sepharose column were buffer-exchanged into buffer A using an Amicon Centriprep 10 (Beverly, MA) at 2200*g*. Protein concentration was determined using a Hewlett Packard 8452A UV/Vis spectrophotometer (Palo Alto, CA) and an extinction coefficient of 0.86 at 280 nm. The protein concentration of the DTPA-conjugated rhG-CSF was confirmed by quantitative amino acid analysis.

Isoelectric Focusing. IEF was performed using Novex pH 3−10 gels (San Diego, CA) with a p*I* 3.5−8.5 performance range. Samples were diluted 1:1 with sample buffer, and 5 μg of protein was loaded into each lane. The gels were run at constant voltages of 100 V for 1 h, 200 V for 2 h, and then 500 V for 0.5 h. All fixing, staining, and destaining procedures were done to the manufacturer's specifications.

Thin-Layer Chromatography. TLC was performed as previously described (Meares et al., 1984) with slight modification. An indium stock solution containing $InCl_3$ with a trace of ^{111}In was prepared in 10 mM HCl, and was used to prepare the following samples: (1) indium added to 100 mM sodium phosphate, pH 6.0; (2) 10 nmol of indium added to 20 nmol of DTPA in 20 mM sodium acetate, pH 5.4; (3) 10 nmol of indium incubated with 2 nmol of rhG-CSF at room temperature for 10 min, followed by addition of 20 nmol of DTPA in 20 mM sodium acetate, pH 5.4; and (4) 10 nmol of indium incubated with 2 nmol of DTPA-conjugated rhG-CSF at room temperature for 10 min, followed by addition of 20 nmol of DTPA in 20 mM sodium acetate, pH 5.4. One microliter of each sample (containing 0.1 nmol of indium) was spotted onto 250 μm thick silica gel (60 Å) on glass backing (Whatman, Clifton, NJ). The TLC plate was developed using 10% (w/v) ammonium acetate in distilled H_2O/methanol (1:1, v/v) as the solvent. The developed plate was then analyzed using a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. MALDI-MS was performed with a Kompact MALDI III mass spectrometer (Kratos Analytical, Ramsey, NJ) fitted with a standard 337 nm nitrogen laser. The spectra were recorded with the analyzer in the linear mode at an accelerating voltage of 20 kV. A sample aliquot containing 15 pmol of protein and 1.0 μL of α-cyano-4-hydroxycinnamic acid was mixed in the sample wells of the probe slide and allowed to air-dry. The laser fluence of the instrument was set at 30 (adjustable over a relative scale of 0−100).

Ion-Spray Mass Spectrometry. Ion-spray mass spectrometry was performed with a Perkin-Elmer Sciex API III mass spectrometer (Norwalk, CT) equipped with an ion-spray interface by the method of flow injection. Samples were diluted in water/acetonitrile/formic acid (50:50:0.1, v/v) and flow-injected into the same solvent flowing at 25 μL/min. The orifice was set at 70 V, and the mass spectrometer was operated in the Q1 mode.

Peptide Mapping. Approximately 0.5 mg of rhG-CSF (standard) or DTPA-conjugated rhG-CSF was dried in a speed vac, reconstituted in 100 μL of 8 M urea, and sonicated for 10 min. After sonication, 10 μL of 1 M Tris-HCl, pH 8.5, and 25 μg of EndoLys-C from a 1 mg/mL stock solution in 10 mM Tris-HCl, pH 8.5, were added. The total volume was adjusted to 200 μL with distilled H_2O, and the proteolytic digestion was carried out for 7 h at room temperature.

Following the hydrolysis with EndoLys-C, the disulfide bonds were simultaneously reduced with 5 μL of 80 mM TBP and alkylated with 10 μL of 40 mM ABD-F (2 mM final concentration) (Kirley, 1989). The reaction mixture was heated at 60 °C for 10 min, and then cooled to room temperature.

Immediately after reduction and alkylation, the generated peptides (200 μL) were injected directly onto a 300 Å pore size C_4 reversed-phase HPLC column (Separations Group, Vydac; Hesperia, CA) equilibrated with solvent A (0.1% TFA in distilled H_2O). Peptide analysis was performed using a Waters HPLC system consisting of two 510 pumps, a WISP 712 autoinjector and a 481 LC spectrophotometer, all controlled through a system interface module by the system software Maxima. The generated peptides were eluted with a linear gradient of 3−76% solvent B (0.1% TFA, 95% acetonitrile) over 115 min. Elution was monitored for absorbance at 215 nm. Individual peptides from the rhG-CSF standard peptide map were collected and identified by amino acid composition analysis and N-terminal sequencing (Souza et al., 1986).

Circular Dichroism. CD spectra were obtained with a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan). Samples (0.078 mg/mL protein) were analyzed at 10 °C in 20 mM sodium acetate, pH 5.4.

In Vivo Biological Activity Assay. Male Syrian hamsters (100 ± 10 g) were dosed (10 μg of protein) by subcutaneous injection. Blood was collected by cardiac puncture after sacrificing by CO_2 asphyxiation. Collected blood samples were analyzed using a Sysmex F800 microcell counter (Kobe, Japan) for WBC concentration.

RESULTS

The DTPA used for conjugation is initially in the dianhydride form, and therefore there exists the potential for undesirable side-reactions such as protein−protein cross-linking. Reaction conditions including initial pH as well as the DTPA dianhydride:rhG-CSF molar ratio were therefore investigated to minimize the formation of such products. RhG-CSF in 100 mM sodium phosphate buffer at pH 6.0, 7.0, or 8.0 was added to DTPA dianhydride-coated tubes to a final molar ratio of 5:1, 50:1, or 500:1 (DTPA dianhydride:rhG-CSF ratio). After passing through G50 spin columns, the reaction products were analyzed by SDS−PAGE (Figure 1). The reactions conducted with an initial pH of 7.0 (lanes 5−7) or 8.0 (lanes 8−10) yielded significant amounts of higher

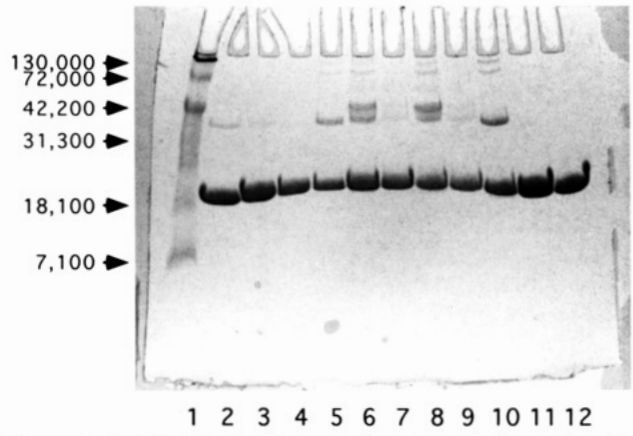

FIGURE 1: Initial pH and molar ratio dependence of the conjugation reaction. SDS−PAGE analysis of the following samples was performed using a 17−27% ISS MiniPlus gel: lane 1, MW markers; lanes 2−4, DTPA dianhydride:rhG-CSF ratio of 5:1, 50:1, and 500:1, respectively, at initial pH 6.0; lanes 5−7, DTPA dianhydride:rhG-CSF ratio of 5:1, 50:1, and 500:1, respectively, at initial pH 7.0; lanes 8−10, DTPA dianhydride:rhG-CSF ratio of 5:1, 50:1, and 500:1, respectively, at initial pH 8.0; and lanes 11 and 12, rhG-CSF from pH 6.0 and pH 8.0 buffer, respectively.

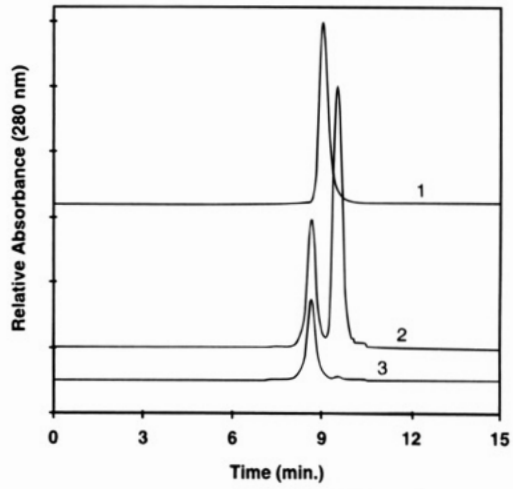

FIGURE 2: Size-exclusion HPLC of rhG-CSF starting material (line 1), and conjugation reaction mixture before (line 2) and after (line 3) the spin column. Elution was monitored for absorbance at 280 nm.

molecular weight species in comparison to the apparent molecular weight observed for unreacted rhG-CSF (lanes 11 and 12). The reactions with an initial pH of 6.0 (lanes 2−4) yielded a single detectable higher molecular weight species. The apparent molecular weight of this species suggests it may be a DTPA-cross-linked protein dimer. This band became less intense with increasing DTPA dianhydride:rhG-CSF molar ratio, and was virtually absent for the 500:1 sample (lane 4). All reaction conditions revealed relatively broad bands with mobilities similar to that of unreacted rhG-CSF. Conjugation of one or more DTPA molecules, in the absence of protein−protein cross-linking, may not have significantly altered the mobility of the protein to be resolved by the applied electrophoretic conditions.

The reaction mixture with an initial pH of 6.0 and a DTPA dianhydride:rhG-CSF molar ratio of 50:1 was analyzed by size-exclusion HPLC both before (Figure 2, line 2) and after (line 3) passing through a G50 spin column. The pre-spin column sample revealed two major peaks with elution times of 8.65 and 9.53 min. The second major peak, which

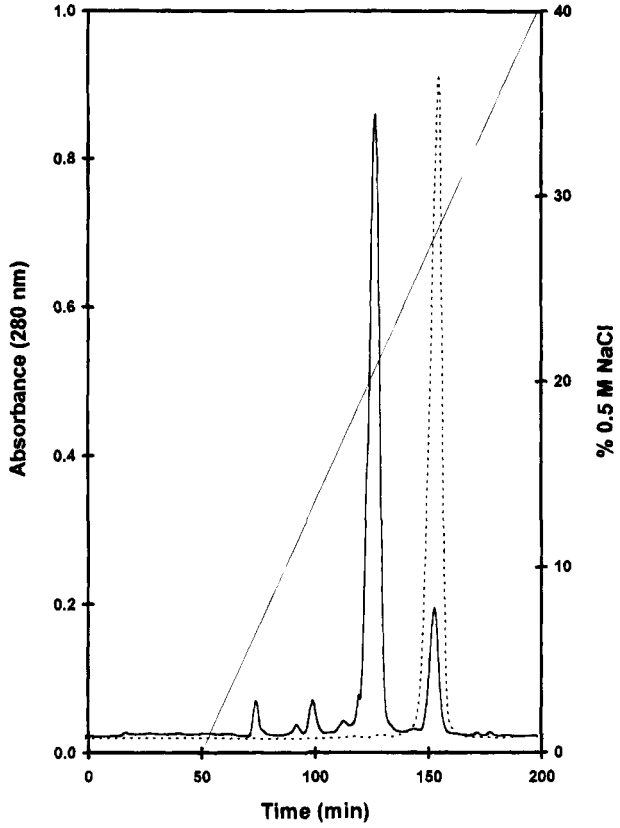

FIGURE 3: Preparative cation-exchange FPLC of rhG-CSF starting material (dashed line) and conjugation reaction mixture (solid line). The light diagonal line indicates buffer B gradient. Elution was monitored for absorbance at 280 nm.

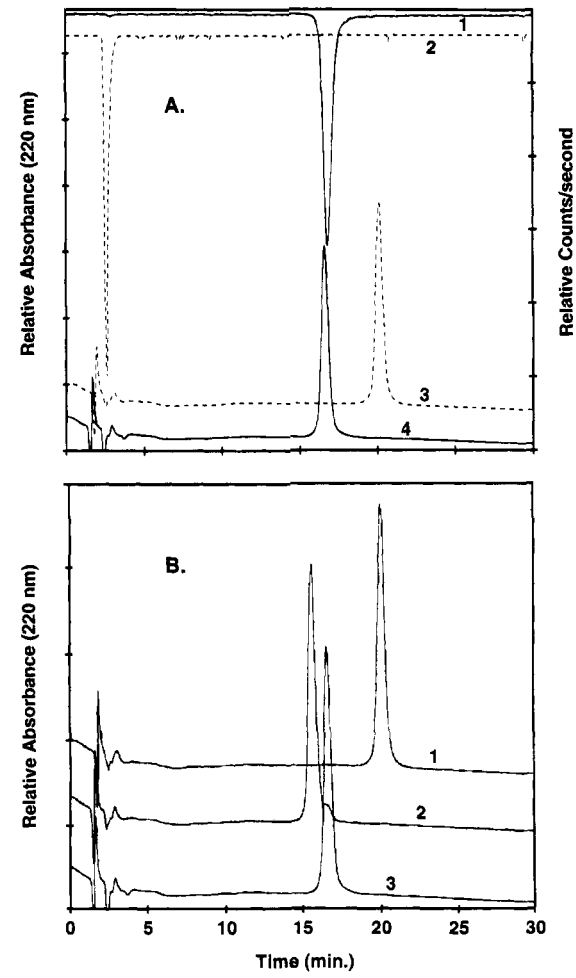

FIGURE 4: (A) Chelation of ^{111}In by the DTPA−rhG-CSF conjugate. RhG-CSF (lines 2 and 3, dashed) or the DTPA−rhG-CSF conjugate (lines 1 and 4, solid) was incubated with ^{111}In for 15 min. An excess of cold indium was then added (In:protein ratio 2:1, mol/mol), and the samples were analyzed by analytical cation-exchange HPLC. Elution was monitored for absorbance at 220 nm (lines 3 and 4, bottom) and radioactivity (lines 1 and 2, inverted). (B) Metal chelation dependence on analytical cation-exchange HPLC analysis of the DTPA−rhG-CSF conjugate. Elution of rhG-CSF (line 1), the DTPA−rhG-CSF conjugate (line 2), and the conjugate incubated with excess $InCl_3$ (In:conjugate ratio 10:1, mol/mol, line 3) was monitored for absorbance at 220 nm. EDTA (1 mM) was added to buffer A.

coeluted with free DTPA (data not shown), was nearly eliminated in the post-spin column sample, indicating successful removal of unbound DTPA from the reaction mixture. The elution time of the remaining major peak was unchanged, and eluted significantly before unreacted rhG-CSF (9.03 min, line 1). This peak likely represents monomeric DTPA-conjugated rhG-CSF. Thus, the behavior of the DTPA-conjugated rhG-CSF on this size-exclusion HPLC column allows it to be resolved from unmodified rhG-CSF. A very minor peak, with an elution time of 7.43 min, represented approximately 1.1% of the combined area with the remaining major peak. The area under this minor peak increased for samples from the 5:1 DTPA dianhydride/rhG-CSF (initial pH 6.0) reaction mixture (data not shown). This peak likely represents the elution of the DTPA-cross-linked rhG-CSF dimer (Figure 1, lane 3).

DTPA-modified rhG-CSF was isolated by preparative cation-exchange FPLC. A diluted reaction mixture originally containing 20 mg of rhG-CSF (initial pH 6.0, DTPA dianhydride:rhG-CSF ratio, 50:1, mol/mol) was directly applied to a HiLoad SP Sepharose HP cation-exchange column and eluted with an increasing salt gradient (Figure 3). A peak representing approximately 13% of the integrated peak areas coeluted with control unreacted rhG-CSF. This result indicates that approximately 13% of the rhG-CSF remained unmodified. A peak eluted between 120 and 130 min contained approximately 84% of the total eluted protein. The shift of this material to elute at a lower salt concentration is in agreement with an increase in negative charge on the protein via conjugation with DTPA. The protein eluting in this peak was isolated and further characterized (hereafter referred to as the DTPA−rhG-CSF conjugate). A small amount of protein eluted at $t < 120$ min, and these peaks may represent possible side reactions, including the conjugation of more than one DTPA molecule per rhG-CSF protein molecule, or possibly aggregates induced by conditions of the reaction. Regardless, the sum of these peaks accounted only for <3% of the total protein.

The pH of the protein sample (initially 6.0) was not maintained during the conjugation reaction. Eventually, an increased acidity of the mixture would result from the production of DTPA by hydrolysis of the dianhydride. An acidic pH may be attained that would inhibit further conjugation. When the buffer strength was decreased to 10 mM sodium phosphate, the efficiency of the reaction was greatly reduced (data not shown). The 0.1 M sodium phosphate buffer therefore likely maintained a favorable pH sufficiently for efficient conjugation.

The chelation of ^{111}In by the DTPA−rhG-CSF conjugate was demonstrated by cation-exchange HPLC (Figure 4A)

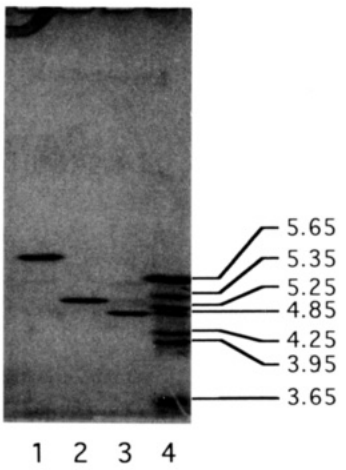

FIGURE 5: Isoelectric focusing of rhG-CSF (lane 1), the DTPA−rhG-CSF conjugate (lane 3), and the conjugate preincubated with excess $InCl_3$ (In:conjugate ratio 10:1, mol/mol, lane 2). Isoelectric point markers were loaded into lane 4. IEF was performed using a Novex pH 3−10 gel.

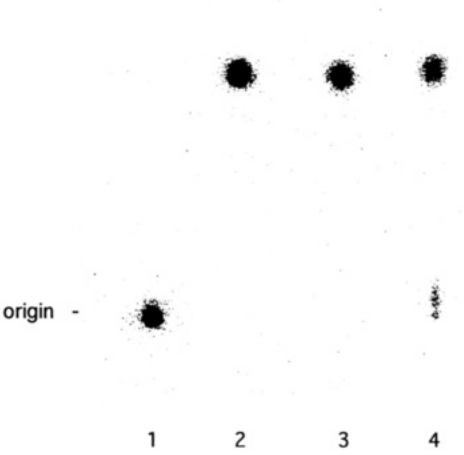

FIGURE 6: TLC assay of the DTPA−rhG-CSF conjugate. Aliquots containing 0.1 nmol of ^{111}In ($InCl_3$ with a trace of ^{111}In) from the following samples were loaded onto a silica gel TLC plate: ^{111}In (lane 1); 10 nmol of ^{111}In added to 20 nmol of DTPA (lane 2); 10 nmol of ^{111}In incubated with 2 nmol of rhG-CSF, followed by addition of 20 nmol of DTPA (lane 3); and 10 nmol of ^{111}In incubated with 2 nmol of DTPA−rhG-CSF conjugate, followed by addition of 20 nmol of DTPA. The developed plate was scanned using a PhosphorImager.

and thin-layer chromatography (see below). For DTPA−rhG-CSF conjugate preincubated with ^{111}In, 99.5% of the radioactivity coeluted from the column with the protein (lines 1 and 4). No detectable radioactivity coeluted with unmodified rhG-CSF preincubated with ^{111}In (lines 2 and 3), indicating the absence of ^{111}In binding by rhG-CSF.

Analysis of the DTPA−rhG-CSF conjugate, without added indium, by cation-exchange HPLC revealed a small shoulder on the higher salt concentration side of the protein peak (Figure 4B, line 2). An aliquot of the preparation was then preincubated with excess $InCl_3$ (In:conjugate ratio of 10:1, mol/mol). This treatment induced all of the protein to coelute with the shoulder of the untreated DTPA−rhG-CSF conjugate (line 3). The shoulder for the untreated sample may therefore represent some contamination (approximately 11%) of this preparation with chelatable metal. The DTPA moiety becomes available for chelation upon conjugation. While the reaction vessels were acid-washed to remove metals, the conjugate may have been exposed to undesirable metals during further handling and instrumental analysis.

The DTPA−rhG-CSF conjugate revealed a single major band of p*I* 4.9 following isoelectric focusing (Figure 5, lane 3). Preincubation of the conjugate with excess $InCl_3$ (In:conjugate ratio of 10:1, mol/mol) shifted the band to p*I* 5.3 (lane 2). The p*I* values of the conjugate, both with and without indium, were lower than that of rhG-CSF, p*I* 6.0 (lane 1).

The stoichiometric molar ratio of DTPA to rhG-CSF for the conjugate was determined by a TLC assay (Meares et al., 1984). Chelation of ^{111}In by DTPA results in migration of all radioactivity near the solvent front (Figure 6, compare lanes 1 and 2). Incubation of ^{111}In (10 nmol) with the DTPA−rhG-CSF conjugate (2 nmol), followed by addition of DTPA, resulted in retention of a portion of the radioactivity at the origin (lane 4). Line graphs of the individual lanes were generated (data not shown), and integation of the peak areas from lane 4 revealed 18% of the radioactivity remained at the origin. The remaining unbound ^{111}In was scavenged by the added DTPA and migrated near the solvent front. Thus, approximately 1.8 nmol of ^{111}In was bound by 2 nmol of the DTPA−rhG-CSF conjugate, indicating a DTPA to rhG-CSF molar ratio of 0.9. Unmodified rhG-CSF did not retain radioactivity at the origin, indicating the absence of ^{111}In binding (lane 3).

The mass of the DTPA−rhG-CSF conjugate was determined by MALDI-MS (Figure 7). The acquired spectrum revealed multiply charged ions in addition to the monoprotonated species. The mass obtained by averaging the peak series was 19 171.7 (±7.3) Da. The calculated mass for a single DTPA conjugated to rhG-CSF is 19 170.8 Da. Therefore, in general agreement with the TLC analysis, the observed mass indicated a DTPA to rhG-CSF molar ratio of 1:1 for the DTPA−rhG-CSF conjugate.

The mass of the DTPA−rhG-CSF conjugate with chelated indium was determined by ion-spray (electrospray) mass spectrometry. Analysis of the conjugate, preincubated with saturating indium (In:conjugate ratio of 10:1, mol/mol), yielded a series of peaks with differing *m*/*z* values. This multiply charged ion series, arising from multiple protonation of the protein, was deconvoluted to produce the molecular weight spectrum shown in Figure 8A. The measured mass of the conjugate with chelated indium was 19 286 (±1.7) Da, which is in agreement with the calculated mass of 19 285.6 Da. For rhG-CSF, the measured mass was 18 798 (±1.8) Da (Figure 8B), in agreement with the calculated mass of 18 798.5 Da.

The location of the conjugated DTPA moiety on the protein was determined by a peptide mapping procedure. Peptide fragments were generated from unmodified rhG-CSF or the DTPA−rhG-CSF conjugate by treatment with EndoLys-C. The peptide fragments were then resolved by reversed-phase HPLC (Figure 9). A peak eluting from the unmodified rhG-CSF sample at 60 min was absent from the DTPA−rhG-CSF conjugate sample. The material eluting in this peak was determined by amino acid composition analysis and N-terminal sequencing to be the N-terminal 17 residues of rhG-CSF. Thus, the corresponding N-terminal peptide fragment from the DTPA−rhG-CSF conjugate was modified, yielding a new partially split double peak eluting at 62 min. Analysis of peptide from each of these partially separated peaks by mass spectrometry revealed the first peak

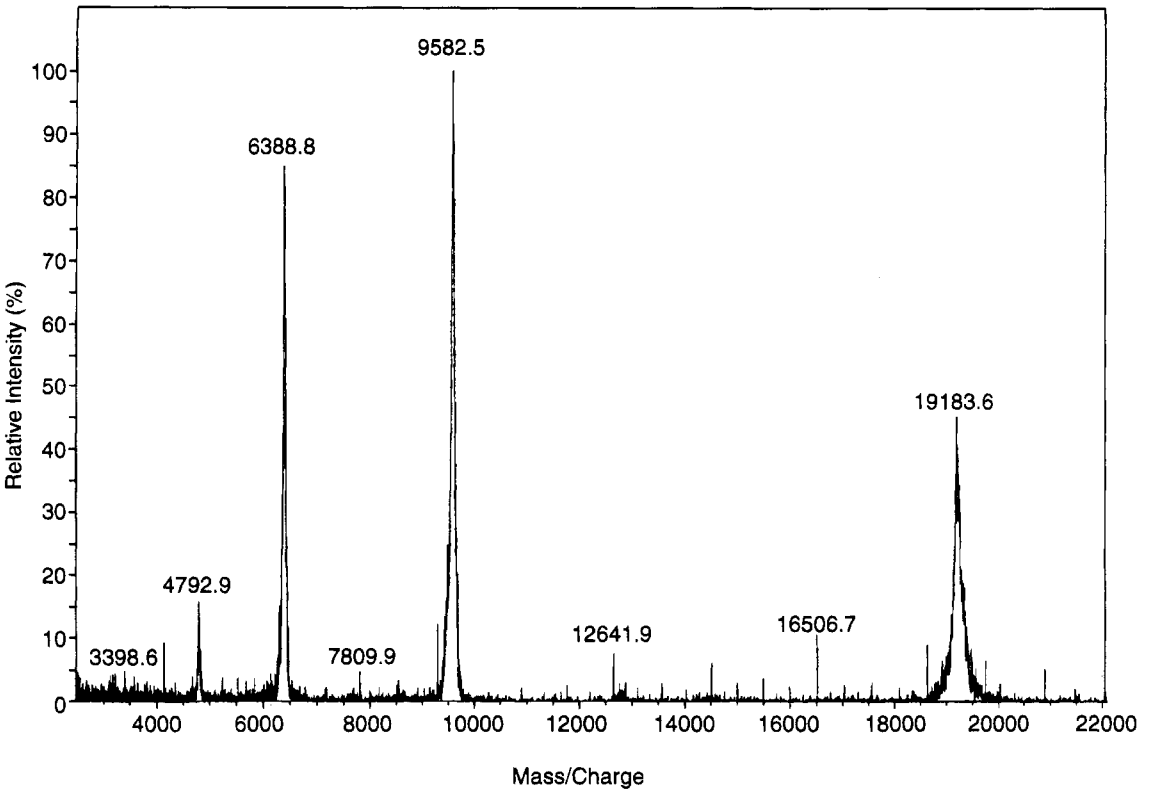

FIGURE 7: MALDI-MS spectrum of the DTPA−rhG-CSF conjugate. The spectrum shows the multiply protonated species (1, 2, 3, and 4 protons attached).

to have the expected mass of the N-terminal peptide with conjugated DTPA (estimated mass of 2160 Da), while the mass of the second peak material suggested conjugated peptide contaminated with iron (estimated mass of 2216 Da, data not shown). Peptide mapping therefore indicated that the conjugated DTPA group is localized to the N-terminal 17 amino acids. This N-terminal peptide contains the N-terminus, one threonine and three serine residues, and a lysine residue. Cleavage of the peptide by EndoLys-C indicates that the lysine is unmodified. Acylation of threonine or serine residues is highly unlikely at pH 6.0. Analysis of the material eluting in the new double peak by N-terminal sequencing indicated a blocked N-terminus (data not shown). Moreover, undigested DTPA−rhG-CSF conjugate subjected to N-terminal sequencing revealed >99% blocked N-terminus (data not shown), indicating the single DTPA moiety on the protein is conjugated to the N-terminus. We therefore postulate that at pH 6.0, only the N-terminus of rhG-CSF is deprotonated and available for nucleophilic attack on a carbonyl carbon atom of the dianhydride to form an amide bond.

Purified and refolded G-CSF possesses a high proportion of α-helical content (approximately 66% at pH 7.5) as has been shown by CD analysis (Wingfield et al., 1988). The extent of secondary structure is sensitive to the solvent pH, where the protein acquires an even higher degree of α-helical content at acidic pH (Lu et al., 1989). The CD spectrum of the DTPA−rhG-CSF conjugate overlays that of unmodified rhG-CSF (Figure 10), each revealing ellipticity minima at 208 and 222 nm. Therefore, the secondary structure (at pH 5.4) is shown here not to be influenced by the conjugation of the chelating group to the N-terminus. Addition of excess indium to saturate all chelating sites on the conjugate (In: conjugate ratio of 10:1, mol/mol) did not change the overall

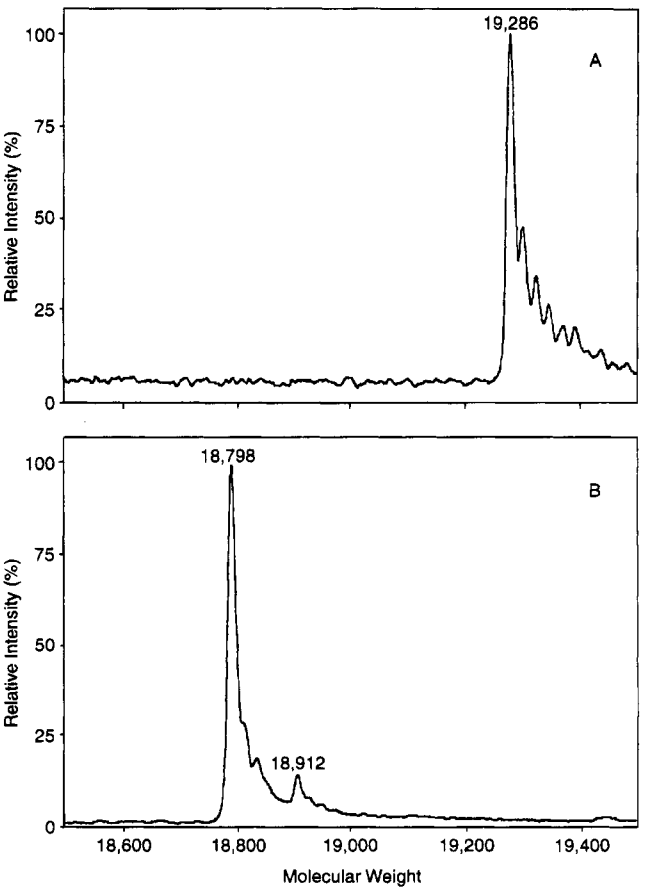

FIGURE 8: Ion-spray mass spectra of the DTPA−rhG-CSF conjugate with chelated indium (A) and of rhG-CSF (B). The conjugate was preincubated with saturating $InCl_3$ (In:conjugate ratio 10:1, mol/mol) before analysis.

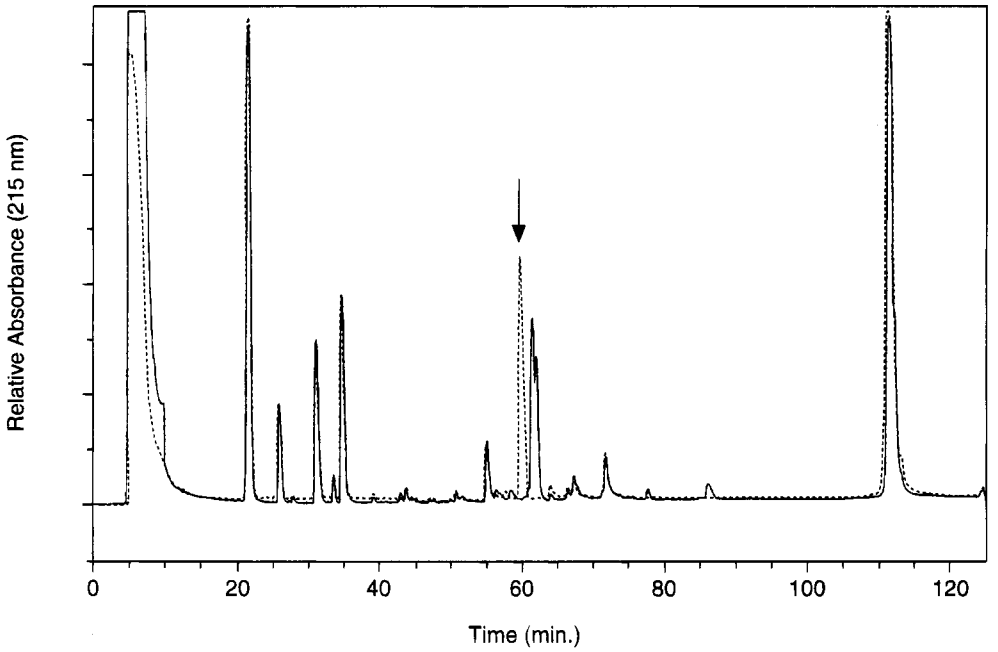

FIGURE 9: Peptide mapping of the DTPA−rhG-CSF conjugate. Peptide fragments generated from the DTPA−rhG-CSF conjugate (solid line) and rhG-CSF (dashed line) by proteolysis were reduced and alkylated, and then resolved by reversed-phase HPLC. Elution was monitored for absorbance at 215 nm. The arrow indicates elution of the N-terminal peptide from the digested rhG-CSF sample.

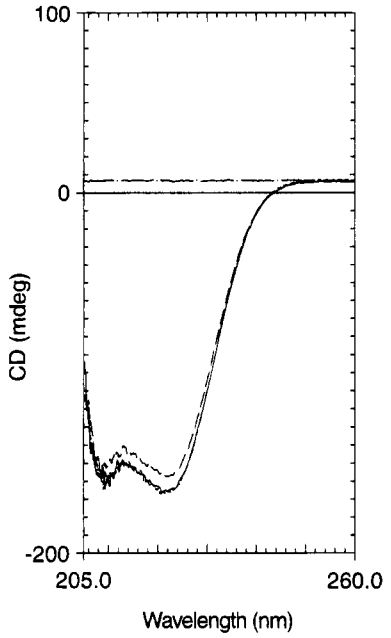

FIGURE 10: CD spectra of the DTPA−rhG-CSF conjugate without (- - -) and with (— —) chelated indium, and of unmodified rhG-CSF (—) and DTPA (— • —). The conjugate with chelated indium was prepared by incubation with saturating $InCl_3$ (In:conjugate ratio 10:1, mol/mol). The samples were analyzed at 10 °C in 20 mM sodium acetate, pH 5.40. The concentration of protein samples was 0.078 mg/mL, and the concentration for the DTPA sample was 4.07 μM.

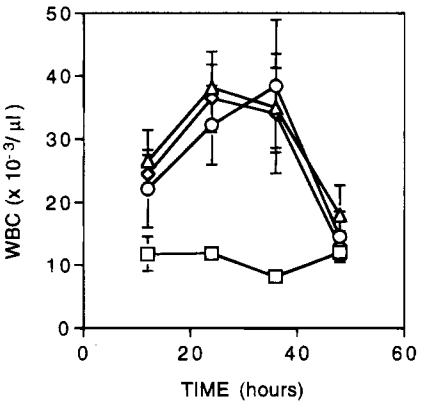

FIGURE 11: Induction of peripheral WBC counts in hamsters by rhG-CSF (○), the DTPA−rhG-CSF conjugate (△), and the conjugate with chelated indium (◇). The conjugate with chelated indium was prepared by preincubation with $InCl_3$ (In:conjugate ratio 10:1, mol/mol). Samples were injected subcutaneously (100 μg of protein/kg body weight). For base line (□), buffer alone was injected. Animals were sacrificed at the indicated time intervals, and collected blood samples were analyzed using a Sysmex F800 microcell counter. The experiment was performed twice, and the results shown are from compilation of the data. Bars represent SD (n = 8−10 for protein samples, and n = 5−6 for base line).

shape of the spectrum, yet caused a slight (~5%) reduction in α-helicity.

Subcutaneous injection of the DTPA−rhG-CSF conjugate into hamsters (100 μg/kg body weight) induced the level of peripheral WBC counts in a manner similar to unmodified rhG-CSF (Figure 11). Injection of the conjugate preincubated with excess indium (In:conjugate ratio of 10:1, mol/mol) also induced a similar response, with maximal WBC levels reached at 24 to 36 h postinjection. Thus, the conjugation of DTPA to rhG-CSF did not significantly alter the observed in vivo activity of the protein, and furthermore, the activity of the conjugate is unchanged after chelation of indium.

DISCUSSION

Several investigators have demonstrated covalent attachment of DTPA to protein by reaction with the dianhydride at pH ≥7.0. However, conjugation of DTPA to insulin yielded a complex mixture of products including cross-linked protein and protein with modified tyrosine residues (Maisano et al., 1992). Reaction of albumin with DTPA dianhydride produced protein molecules with multiple chelating groups attached (Lauffer & Brady, 1985; Ogan et al., 1987). IgG

conjugates have been prepared with an average of 0.7 DTPA group per protein molecule (Hnatowich et al., 1983), and it has been emphasized that these preparations are heterogeneous, where many molecules bear more and less DTPA groups than the average number (Hnatowich & McGann, 1987).

RhG-CSF contains four lysine residues in addition to the N-terminus for a total of five potential reactive amino groups. Each protein molecule also contains several serine, threonine, and tyrosine residues as potential sights for acylation (Maisano et al., 1992). The present studies show that by initiating the reaction in buffer at pH 6.0, a single DTPA can be conjugated to the protein, and the conjugation is specific to the N-terminus. Furthermore, the reaction is efficient in the 84% of the total protein in the reaction mixture is N-terminally conjugated, and this product can be highly purified.

Any functional importance of the N-terminal region of rhG-CSF remains to be elucidated. While the overall homology of the polypeptide sequences of human and murine G-CSF is ~73%, the N-terminal sequences are highly divergent (Tsuchiya et al., 1986). Deletion of the N-terminal 4, 6, 7, and 11 amino acids of rhG-CSF did not reduce the activity of the protein in vitro (Kugo et al., 1989). Monoclonal antibodies recognizing the N-terminal 16 residues of rhG-CSF failed to significantly neutralize bioactivity in vitro (Layton et al., 1991). The present results show that conjugation of DTPA specifically to the N-terminus did not affect the in vivo biological activity of rhG-CSF. Furthermore, while chelation of indium by the DTPA−rhG-CSF conjugate caused a slight reduction in α-helicity, this did not affect the observed bioactivity.

The conjugation of a single DTPA moiety would add three amino groups and four carboxylic acid groups to rhG-CSF. The added negative charge likely caused the decreased retention time of the protein during cation-exchange chromatography. During isoelectric focusing, the effect of the added carboxylic acid groups may have been partially nullified by the amino groups of DTPA. Regardless, the conjugation of DTPA, concomitant with the loss of the N-terminal free amino group, substantially decreased the p*I* of the protein. Chelation of indium by the DTPA−rhG-CSF conjugate caused a slight increase in retention time during cation-exchange chromatography relative to that of the conjugate without chelated metal. Furthermore, chelated indium slightly increased the p*I* of the conjugate. The conjugated DTPA is likely a heptadentate ligand, binding the indium through all three amino groups and the four carboxylic acid groups (Maecke et al., 1989). Interaction of these functional groups with indium may have reduced their effects of increased negative charge/acidity on the protein. The remaining difference in retention time and p*I* compared to the unmodified rhG-CSF may be primarily due to the loss of the free primary N-terminal amino group. The characteristic retention times and isoelectric points of the DTPA−rhG-CSF conjugate without and with chelated metal may be used to monitor metal contamination of the conjugate preparation. Furthermore, these analyses may be used to monitor metal labeling of the conjugate. Similar modifications to the p*I* of an IgG antibody resulting from DTPA conjugation and subsequent indium labeling have recently been observed (Legendre et al., 1993).

Attempts to radioiodinate G-CSF using standard chloramine-T, Bolton−Hunter reagent, or Iodogen iodination protocols were reported as unsuccessful (Avalos et al., 1990; Nicola & Metcalf, 1984). However, iodinated G-CSF preparations with specific activities in the range of 1×10^5 to 1×10^6 cpm/ng were generated using lactoperoxidase (Avalos et al., 1990). Unfortunately, the preparations revealed reduced activity (≥50%) as well as rapid loss of receptor binding capacity within 72 h. Iodinated G-CSF prepared using a chloramine-T two-phase method (Tejedor & Ballesta, 1982), and with specific activities ranging from 2.5×10^5 to 4.5×10^5 cpm/ng, revealed no loss of colony-stimulating activity (Nicola & Metcalf, 1984). The present studies describe a method for preparing a well-defined DTPA−rhG-CSF conjugate in large quantity, and which retains full in vivo bioactivity either with or without chelated indium. Theoretically, the conjugate can be labeled with ^{111}In (152 000 Ci/g) to a maximum specific activity of $\sim 2.0 \times 10^6$ dpm/ng. Moreover, the conjugated DTPA should offer wide versatility in the choice of metallic radionuclides for labeling (Meares & Goodwin, 1984).

The three-dimensional structure of rhG-CSF has recently been determined by X-ray crystallography (Hill et al., 1993). G-CSF belongs to a structural class of cytokines which share a common motif of a four α-helix bundle with two long crossover connections (Hill et al., 1993). Other cytokines in this group include granulocyte/macrophage-colony-stimulating factor (Diederichs et al., 1991), growth hormone (De Vos et al., 1992), interferon β (Senda et al., 1992), IL-2 (Bazan, 1992), and IL-4 (Powers et al., 1992). Conjugation of DTPA to such related proteins is currently under investigation.

The IL-2 receptor (IL-2R) is constitutively overexpressed in various hematologic malignancies including adult T-cell leukemia (Uchiyama et al., 1985), hairy cell leukemia (Trentin et al., 1992), chronic lymphocyte leukemia (Rosolen et al., 1989), Hodgkin's disease (Strauchen & Breakstone, 1987), and non-Hodgkin's lymphoma (Grant et al., 1986). Lymphocytes involved in several autoimmune diseases, including rheumatoid arthritis (Lemm & Warnatz, 1986) and allograft rejection (Waldmann, 1989), also overexpress the IL-2R. This receptor has therefore been actively pursued as a target for cytotoxic therapy. Recombinant fusion toxins have been produced in which the cell-binding domain of *Pseudomonas* exotoxin (Lorberboum-Galski et al., 1988a) or the receptor-binding domain of diphtheria toxin (Williams et al., 1987) has been replaced with IL-2. These fusion proteins are specifically cytotoxic to cells that express the high-affinity IL-2R (Lorberboum-Galski et al., 1988b; Williams et al., 1990). A recently described *Pseudomonas* exotoxin/IL-4 chimeric protein may also prove useful for the treatment of autoimmune diseases, allograft rejections, and many hematologic malignancies where cells express elevated levels of IL-4 receptor (Puri et al., 1994). A diphtheria toxin-related human G-CSF fusion protein has also recently been constructed which may have usefulness in the study and treatment of acute myeloblastic leukemia (AML) (Chadwick et al., 1993), where the presence of a receptor specific for G-CSF on AML cells has been demonstrated (Budel et al., 1989). Conjugation of DTPA to IL-2, IL-4, and also rhG-CSF may also have potential use in diagnosis, imaging, and/or treatment of leukemia and related diseases. Chelatable radiometals for cytotoxic therapy may include

^{212}Bi, ^{211}At, and ^{90}Y. Indeed, antibodies to the IL-2R α chain, and bearing radioisotopes ^{212}Bi and ^{90}Y via conjugated bifunctional chelates, are being examined by several investigators (Junghans et al., 1993; Parenteau et al., 1992; Kozak et al., 1990) for cytotoxicity toward alloreactive T-cell lines, and for potential radiotherapy.

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REFERENCES

Avalos, B. R., Gasson, J. C., Hedvat, C., Quan, S. G., Baldwin, G. C., Weisbart, R. H., Williams, R. E., Golde, D. W., & DiPersio, J. F. (1990) *Blood 75*, 851−857.

Bazan, J. F. (1992) *Science 257*, 410−413.

Budel, L. M., Touw, I. P., Delwel, R., & Löwenberg, B. (1989) *Blood 74*, 2668−2673.

Chadwick, D. E., Williams, D. P., Niho, Y., Murphy, J. R., & Minden, M. D. (1993) *Leuk. Lymphoma 11*, 249−262.

De Vos, A. M., Ultsch, M., & Kossiakoff, A. A. (1992) *Science 255*, 306−312.

Diederichs, K., Boone, T., & Karplus, P. A. (1991) *Science 254*, 1779−1782.

Grant, B. W., Platt, J. L., Jacob, H. S., & Kay, N. E. (1986) *Leuk. Res. 10*, 1271−1278.

Hill, C. P., Osslund, T. D., & Eisenberg, D. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5167−5171.

Hnatowich, D. J., & McGann, J. (1987) *Int. J. Radiat. Appl. Instrum., Part B 14*, 563−568.

Hnatowich, D. J., Layne, W. W., Childs, R. L., Lanteigne, D., David, M. A., Griffin, T. W., & Doherty, P. W. (1983a) *Science 220*, 613−615.

Hnatowich, D. J., Childs, R. L., Lanteigne, D., & Najafi, A. (1983b) *J. Immunol. Methods 65*, 147−157.

Junghans, R. P., Dobbs, D., Brechbiel, M. W., Mirzadeh, S., Raubitschek, A. A., Gansow, O. A., & Waldmann, T. A. (1993) *Cancer Res. 53*, 5683−5689.

Kirley, T. K. (1980) *Anal. Biochem. 180*, 231−236.

Kozak, R. W., Fitzgerald, D. P., Atcher, R. W., Goldman, C. K., Nelson, D. L., Gansow, O. A., Pastan, I., & Waldmann, T. A. (1990) *J. Immunol. 144*, 3417−3423.

Kuga, T., Komatsu, Y., Yamasaki, M., Sekine, S., Miyaji, H., Nishi, T., Sato, M., Yokoo, Y., Asano, M., Okabe, M., Morimoto, M., & Itoh, S. (1989) *Biochem. Biophys. Res. Commun. 159*, 103−111.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Lauffer, R. B., & Brady, T. J. (1985) *Magn. Reson. Imaging 3*, 11−16.

Layton, J. E. (1992) *Growth Factors 6*, 179−186.

Layton, J. E., Morstyn, G., Fabri, L. J., Reid, G. E., Burgess, A. W., Simpson, R. J., & Nice, E. C. (1991) *J. Biol. Chem. 266*, 23815−23823.

Legendre, J. M., Bergot, A., Turzo, A., & Morin, P. P. (1993) *Pathol. Biol. 41*, 169−171.

Lemm, G., & Warnatz, H. (1986) *Clin. Exp. Immunol. 64*, 71−79.

Lorberboum-Galski, H., FitzGerald, D., Chaudhary, V., Adhya, S., & Pastan, I. (1988a) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1922−1926.

Lorberboum-Galski, H., Kozak, R. W., Waldmann, T. A., Bailon, P., FitzGerald, D. J., & Pastan, I. (1988b) *J. Biol. Chem. 263*, 18650−18656.

Lu, H. S., Boone, T. C., Souza, L. M., & Lai, P. H. (1989) *Arch. Biochem. Biophys. 268*, 81−92.

Maecke, H. R., Riesen, A., & Ritter, W. (1989) *J. Nucl. Med. 30*, 1235−1239.

Maisano, F., Gozzini, L., & de Haen, C. (1992) *Bioconjugate Chem. 3*, 212−217.

Meares, C. F., & Goodwin, D. A. (1984) *J. Protein Chem. 3*, 215−228.

Meares, C. F., McCall, M. J., Reardan, D. T., Goodwin, D. A., Diamanti, C. I., & McTigue, M. (1984) *Anal. Biochem. 142*, 68−78.

Mukkala, V. M., Mikola, H., & Hemmilä, I. (1989) *Anal. Biochem. 176*, 319−325.

Nicola, N. A., & Metcalf, D. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3765−3769.

Ogan, M. D., Schmiedl, U., Moseley, M. E., Grodd, W., Paajanen, H., & Brasch, R. C. (1987) *Invest. Radiol. 22*, 665−671.

Parenteau, G. L., Dirbas, F. M., Garmestani, K., Brechbiel, M. W., Bukowski, M. A., Goldman, C. K., Clark, R., Gansow, O. A., & Waldmann, T. A. (1992) *Transplantation 54*, 963−968.

Penefsky, H. S. (1979) *Methods Enzymol. 56*, 527−530.

Powers, R., Garrett, D. S., March, C. J., Frieden, E. A., Gronenborn, A. M., & Clore, G. M. (1992) *Science 256*, 1673−1677.

Puri, R. K., Mehrotra, P. T., Leland, P., Kreitman, R. J., Siegel, J. P., & Pastan, I. (1994) *J. Immunol. 152*, 3693−3700.

Rosolen, A., Nakanishi, M., Poplack, D. G., Cole, D., Quinones, R., Raeman, G., Trepel, J. B., Cotelingam, J. D., Sausville, E. A., Marti, G. E., Jaffe, E. S., Neckers, L. M., & Colamonici, O. R. (1989) *Blood 73*, 1968−1972.

Senda, T., Shimazu, T., Matsuda, S., Kawano, G., Shimizu, H., Nakamura, K. T., & Mitsui, Y. (1992) *EMBO J. 11*, 3193−3201.

Souza, L. M., Boone, T. C., Gabrilove, J., Lai, P. H., Zsebo, K. M., Murdock, D. C., Chazin, V. R., Bruszewski, J., Lu, H., Chen, K. K., Barendt, J., Platzer, E., Moore, M. A. S., Mertlesmann, R., & Welte, K. (1986) *Science 232*, 61−65.

Strauchen, J. A., & Breakstone, B. A. (1987) *Am. J. Pathol. 126*, 506−512.

Tejedor, F., & Ballesta, J. P. G. (1982) *Anal. Biochem. 127*, 143−149.

Trentin, L., Zambello, R., Benati, C., Cassatella, M., Agostini, C., Bulian, P., Adami, F., Carra, G., Pizzolo, G., & Semenzato, G. (1992) *Cancer Res. 52*, 5223−5228.

Tsuchiya, M., Asano, S., Kaziro, Y., & Nagata, S. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7633−7637.

Uchiyama, T., Hori, T., Tsudo, M., Wano, Y., Umadome, H., Tamori, S., Yodoi, J., Maeda, M., Sawami, H., & Uchino, H. (1985) *J. Clin. Invest. 76*, 446−453.

Waldmann, T. A. (1989) *Annu. Rev. Biochem. 58*, 875−911.

Williams, D. P., Parker, K., Bacha, P., Bishai, W., Borowski, M., Genbauffe, F., Strom, T. B., & Murphy, J. R. (1987) *Protein Eng. 1*, 493−498.

Williams, D. P., Snider, C. E., Strom, T. B., & Murphy, J. R. (1990) *J. Biol. Chem. 265*, 11885−11889.

Wingfield, P., Benedict, R., Turcatti, G., Allet, B., Mermod, J. J., DeLamanter, J., Simona, M. G., & Rose, K. (1988) *Biochem. J. 256*, 213−218.

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