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Development of glycosylated human interleukin-1*α*, neoglyco IL-1*α*, by coupling with D-galactose monosaccharide: synthesis and purification

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In order to develop glycosylated cytokine, recombinant human IL-1*α* was chemically modified with galactose monosaccharide. Galactose with C9 spacer, 8-(hydrazinocarbonyl)octyl *β*-D-galctopyranoside (3), was synthesized by glycosylation of C9 spacer, methyl 9-hydroxynonanoate, with acetobromogalactose, followed by deacetylation and hydrazidation. Total yield of 3 was 43.6% in three steps. Compound 3 was coupled to IL-1*α* by the acyl azide method. The glycosylated IL-1 was purified by anion-exchange chromatography, and galactose coupled to IL-1 was confirmed by *R. communis* lectin blotting. Based on the molecular weight, the average number of carbohydrate molecules introduced per molecule of IL-1*α* was estimated to be 9.1.

***Keywords*: neoglycoprotein, interleukin 1, cytokine**

Introduction

Glycoproteins, proteins that contain covalently bound carbohydrates, are widely distributed in animals, plants and microorganisms. Several approaches have been taken in order to investigate the role and contribution of carbohydrates to the functions mediated by glycoproteins in a variety of biological reactions. These are divided into several classes; (1) obtaining natural variants; (2) treating native glycoproteins with enzymes to alter or perform partial removal of the carbohydrate residues; and (3) preparing synthetic glycoproteins, that is, neoglycoproteins, proteins chemically or enzymatically coupled with carbohydrates.

The great advantage of synthesizing neoglycoproteins is that any chemically synthesized carbohydrates can be introduced into proteins. A neoglycoprotein was first prepared to raise carbohydrate-directed antibodies [1]. In order to examine the immunogenicity of the carbohydrate, neoglycoproteins conjugated with carbohydrates of *Streptococcus pneumoniae* [2] and species of *Salmonella* [3] have been prepared. These neoglycoproteins have also been used to determine the specificities of carbohydrate-specific antibodies [4]. In addition, neoglycoproteins have been used to detect lectins in tissues or cell surfaces [5], and may be used as affinity ligands to isolate carbohydrate-binding proteins [6]. Neoglycoproteins may also be used as soluble inhibitors of glycoconjugate-mediating processes, such as cell–cell contact [7].

Several methods have been developed to synthesize neoglycoproteins, including the imidate method [1], the reductive amination method [8] and the acyl azide method [9]. In a previous study we have chemically introduced D-mannose dimers into recombinant human interleukin-1α (rhIL-1α) by the acyl azide method [10] and biological activities *in vitro* and *in vivo* of the glycosylated IL-1s were investigated [11–13]. The method appeared to be applicable to biologically active proteins, because the reaction could be conducted under mild conditions, pH 9 at 0 °C.

IL-1 is a cytokine produced mainly by macrophages and monocytes. IL-1 exhibits a variety of biological activities, and plays an important role in immunological and inflammatory reactions [14]. By introduction of a carbohydrate into IL-1α, there are several possibilities of expected effectiveness: (1) a particular activity may be inhibited or augmented; (2) IL-1 with less side effects may be obtained; (3) an IL-1 inhibitor may be obtained; or (4) IL-1 with a novel activity may be obtained. In this study we synthesized and purified rhIL-1α into which galactose had been chemically introduced.

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Materials and methods

General procedures

Melting points were determined with a Yanagimoto MP-S2 micro melting point apparatus and are uncorrected. Solutions were concentrated in a rotary evaporator below 50 °C under vacuum. Optical rotations were measured with a JASCO DIP-140 automatic digital polarimeter in a 0.5 dm tube. IR spectra were recorded with a Perkin-Elmer 1600 Series FTIR spectrometer. ^{1}H NMR spectra were recorded at 270 MHz with a JEOL JNM-ES-270, or at 500 MHz with a JEOL α-500 spectrometer, ^{13}C NMR spectra were recorded at 67.5 MHz with a JEOL JNM-ES-270, or at 125 MHz with a JEOL α-500 spectrometer. Tetramethylsilane was used as an internal standard. Chemical shifts are given on the δ scale. TLC was performed on precoated silica gel plates 0.25 mm thick (Kieselgel 60F_{254}, Merck). Detection was effected with H_2SO_4 or by UV irradiation at 254 nm. Column chromatography was performed on Silica Gel BW-820MH (Fuji-Silysia Chemical Ltd, Nagoya). Solvent combinations for elution of a column chromatography and the developing solvent on TLC are given as v/v.

8-(Methoxycarbonyl)octyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **(1)**

To a solution of methyl 9-hydroxynonanoate [10] (0.97 g, 5.15 mmol) and 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (2.08 g, 5.06 mmol) in distilled nitromethane (42 ml), mercuric cyanide (1.24 g, 4.90 mmol), and Drierite (4.2 g) were added. The mixture was stirred for 24 h, at room temperature and filtered. The residue was washed with dichloromethane, and the combined filtrate and washings were concentrated to a syrup which was dissolved in dichloromethane. The dichloromethane solution was washed with water, 10% KBr solution (×2), and water, dried ($MgSO_4$), and concentrated to afford a syrup which was chromatographed on a column of silica gel with chloroform–acetone (50:1–6:1) as eluent. Evaporation of the solvent gave **1** (1.38 g, 52.7%) as a syrup: $[\alpha]_D^{22} - 0.7°$ (*c* 1.07, $CHCl_3$); TLC (20:1 chloroform: acetone) Rf 0.48; IR (neat) 1752 and 1223 cm^{-1} (ester); ^{1}H NMR ($CDCl_3$) δ 1.25–1.67 [m, 12H, $CH_2(CH_2)_6CH_2$], 1.99, 2.05(×2), 2.15 (3s, 12H, 4AcO), 2.30 (t, 2H, J = 7.5 Hz, CH_2COOMe), 3.47, 3.88 (m, 2H, CH_2O), 3.67 (s, 3H, MeO), 3.90 (m, 1H, H-5), 4.13 (dd, 1H, $J_{5,6a}$ = 7.2 Hz, $J_{6a,6b}$ = 11.2 Hz, H-6a), 4.19 (dd, 1H, $J_{5,6b}$ = 6.4 Hz, H-6b), 4.46 (d, 1H, $J_{1,2}$ = 8.1 Hz, H-1), 5.02 (dd, 1H, $J_{2,3}$ = 10.4 Hz, $J_{3,4}$ = 3.5 Hz, H-3), 5.20 (dd, 1H, H-2), and 5.39 (dd, 1H, $J_{4,5}$ = 0.9 Hz, H-4); ^{13}C NMR ($CDCl_3$) δ 20.6, 20.7 (×2), 20.8 (4CO*Me*), 24.9 25.7, 29.1 (×2), 29.2 29.4 [$CH_2(CH_2)_6CH_2$], 34.1 (CH_2COOMe), 51.4 (MeO), 61.3 (C-6), 67.1 (C-4), 69.0 (C-2), 70.2 (CH_2O), 70.6 (C-5), 71.0 (C-3), 101.4 (C-1), 169.4, 170.2, 170.3, 170.4 (4*C*OMe), and 174.3 (*C*OOMe).

Anal. Calcd for $C_{24}H_{38}O_{12}$·1/2 H_2O (527.57): C, 54.64; H, 7.45. Found: C, 54.43; H, 7.48.

8-(Methoxycarbonyl)octyl β-D-Galactopyranoside **(2)**

0.5 M Methanolic sodium methoxide (1 ml) was added dropwise with stirring to a chilled solution of **1** (5.43 g, 10.47 mmol) in dry methanol (100 ml). After being stirred overnight at room temperature, the mixture was neutralized with Amberlite IR-120(H^+) resin, filtered, and concentrated to afford a syrup which was crystallized from ethyl acetate. Recrystallization from the same solvent gave **2** (3.23 g, 88.0%) as white crystals: mp 104–105 °C; $[\alpha]_D^{26} - 7.1°$ (*c*. 1.13, MeOH); TLC (3:1 chloroform-methanol) Rf 0.60; IR (KBr) 3600–3100 (OH), and 1731 and 1062 cm^{-1} (ester); ^{1}H NMR (CD_3OD) δ 1.25–1.67 [m, 12H, $CH_2(CH_2)_6CH_2$], 2.31 (t, 2H, J = 7.3 Hz. CH_2COOMe), 3.46 (dd, 1H, $J_{2,3}$ = 9.8 Hz, $J_{3,4}$ = 3.1 Hz, H-3), 3.49 (m, 2H, H-2,5), 3.54 3.89 (m, 2H, CH_2O), 3.65 (s, 3H, MeO), 3.73 (m, 2H, H-6a,6b), 3.83 (m, 1H, H-4), and 4.20 (δ, 1H, $J_{1,2}$ = 7.3 Hz, H-1); ^{13}C NMR (CD_3OD) δ 26.0, 27.0, 30.1, 30.3, 30.4, 30.8 [$CH_2(CH_2)_6CH_2$], 34.8 (CH_2COOMe), 52.0 (MeO), 62.4 (C-6), 70.3 (C-4), 70.8 (CH_2O), 72.6 (C-2), 75.0 (C-3), 76.5 (C-5), 105.0 (C-1), and 176.0 (*C*OOMe).

Anal. Calcd for $C_{16}H_{30}O_8$(350.41): C, 54.84; H, 8.63. Found: C, 54.59; H, 8.64.

8-(Hydrazinocarbonyl)octyl β-D-Galactopyranoside **(3)**

A mixture of **2** (1.00 g, 2.85 mmol) and hydrazine hydrate (3.5 ml, 72.0 mmol) in distilled methanol (50 ml) was stirred for 3 days at room temperature. After several hours, white crystals began to precipitate. The crystals were collected by filtration, washed with methanol and dried in the air to afford **3** (0.94 g, 94.0%) as white crystals: mp 203–204 °C: $[\alpha]_D^{22} - 10.9°$ (*c* 1.07, DMSO); TLC (3:1 chloroform: methanol) Rf. 0.26; IR (KBr) 3500–3150 (OH, NH, NH_2), and 1636 and 1534 cm^{-1} (amide); ^{1}H NMR (pyridine-d_5) δ 1.05–1.80, [m, 12H, $CH_2(CH_2)_6CH_2$], 2.49 (t, 2H, J = 7.3 Hz, $CH_2CONHNH_2$), 3.66, 4.11 (m, 2H, CH_2O), 4.08 (m, 1H, H-5), 4.18 (dd, 1H, $J_{2,3}$ = 9.2 Hz, $J_{3,4}$ = 3.7 Hz, H-3), 4.48 (m, 3H, H-2, 6a, 6b), 4.58 (m, 1H, H-4), and 4.77 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1): ^{13}C NMR(pyridine-d_5) δ 25.6, 26.4, 29.5, 29.6(×2), 30.3 [$CH_2(CH_2)_6CH_2$], 34.9 ($CH_2CONHNH_2$), 62.5 (C-6), 69.7 (CH_2O), 70.3 (C-4), 72.6 (C-2), 75.4 (C-3), 77.0 (C-5), 105.3 (C-1), and 176.0 ($CONHNH_2$).

Anal. Calcd for $C_{15}H_{30}N_2O_7$ (350.42): C, 51.42; H, 8.63; N, 7.99. Found: C, 51.15; H, 8.60; N, 7.94.

Coupling of **3** with rhIL-1α

Recombinant human IL-1α (rhIL-1α) was provided by Dr M. Yamada of Dainippon Pharmaceutical Co. (Osaka, Japan). Compound **3** (4.0 mg, 11.5 μmol) was dissolved in water (300 μl) and the solution chilled on ice. To the chilled solution, cold 4 M HCl (45 μl) and 2 M sodium nitrite (28.8 μmol) were added. After the solution was kept at room temperature for 15 min, 2 M ammonium sulfamate (28.8 μmol) was added and the mixture was kept at room

temperature for 15 min in order to destroy excess HNO_2. This mixture (containing the acyl azide) was added to ice-cooled 0.4 M sodium borate buffer (pH 10.0) containing 1.984 mg of rhIL-1α (500 μl). The pH was quickly adjusted to 9.0 with 4 N NaOH with stirring for 60 min at room temperature. The reaction mixture was then filtrated with 0.2 μm nylon membrane filter and desalted with 20 mM Tris-HCl buffer (pH 7.0) with HiTrap Desalting column (Pharmacia). The rhIL-1α treated with the same manner without compound **3** was used as control (mock treated) IL-1α.

Purification of galactose-introduced IL-1α

Purification was carried out at room temperature employing the FPLC system (Pharmacia). The desalted sample was loaded onto an anion-exchange chromatography column (Mono Q, Pharmacia) equilibrated with 20 mM Tris-HCl buffer (pH 7.0) at flow rate of 1 ml min^{-1}, eluted with a 30 ml liner NaCl gradient (0–0.5 M) in the same buffer, and fractions (1 ml) were collected. The buffer of fractions containing galactose-introduced IL-1α was exchanged to PBS with HiTrap and concentrated in Centriplus-3 (amicon, M_r 30 000 cut-off). The final yield of Gal-IL-1α was 36.0%.

Electrophoresis

Analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [15] on 15% polyacrylamide gel in the presence of 0.1% SDS using a vertical slab minigel apparatus. Protein bands were visualized with Coomassie brilliant blue at room temperature for 2 h and decolorized with 10% acetate solution overnight. Molecular weight of galactose-introduced IL-1α was determined by comparison of the electrophoretic mobility with those of standard molecular weight markers (phosphorylase b, M_r 94 000; bovine serum albumin, M_r 67 000; ovalbumin, M_r 43 000; carbonic anhydrase, M_r 30 000; soybean trypsin inhibitor, M_r 20 100; α-lactalbumin, M_r 14 400).

Lectin blotting

Size fractionated proteins were transfered from gels to Immobilon polyvinylidene difluoride membrane (Milipore Corporation, Bedford, MA) using a semidry apparatus (Marysol, Tokyo, Japan) for 2 h with a current of 0.2 A at room temperature. The membrane was washed with PBS containing 0.02% Tween 20 and treated with 1% BSA at 4 °C overnight. The membrane was then treated with 100 ng ml^{-1} horse-radish peroxidase (HRP)-conjugated RCA120 for 1 h at room temperature, washed three times and treated with the substrate solution (0.6 mg ml^{-1} 3,3′-diamino-benzidine tetrahydrochloride and 0.01% H_2O_2 in 0.1 M Tris-HCl, pH 7.4).

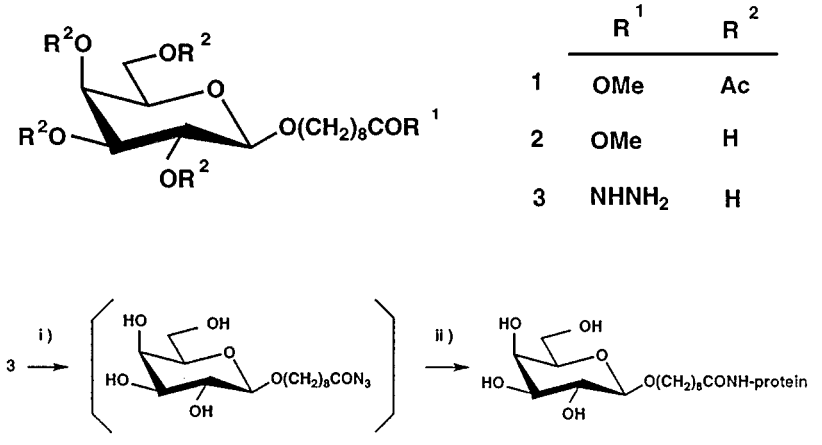
Determination of protein content

The amount of protein was determined using a protein assay kit (Bio-rad, Richmond, CA) with bovine serum albumin as a standard.

Results and discussion

Synthesis of 8-(Hydrazinocarbonyl)octyl β-D-galactopyranoside (3)

A mixture of methyl 9-hydroxynonanoate,[10] 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide, mercuric cyanide and Drierite in dry nitromethane was stirred for 24 h at room temperature. After purification with column chromatography, compound **1** was obtained as a syrup in 52.7% yield. ^{1}H NMR spectrum of **1** showed the signal due to H-1 at δ 4.46 ($J_{1,2}$ = 8.1 Hz). The coupling constant of H-1, H-2 indicates the stereochemistry of the newly formed glycosidic bond to be β. Deacetylation of **1** by Zemplen's



Scheme 1. i) $NaNO_2$, HCl ii) IL-1*a* (H_2N-protein)

method (sodium methoxide-methanol) gave **2** which was easily crystallized from ethyl acetate as white crystals in 88.0% yield. The ^{1}H NMR spectrum of **2** showed the signal due to H-1 at δ 4.20 ($J_{1,2} = 7.3$ Hz). The signal due to a methyl ester appeared at δ 3.67. Treatment of **2** with hydrazine hydrate in methanol afforded **3** which was easily precipitated in the reaction mixture as white crystals in 94.0% yield. The ^{1}H NMR spectrum of **3** showed the signal due to H-1 at δ 4.77 ($J_{1,2} = 7.9$ Hz), but no resonance due to a methyl ester group.

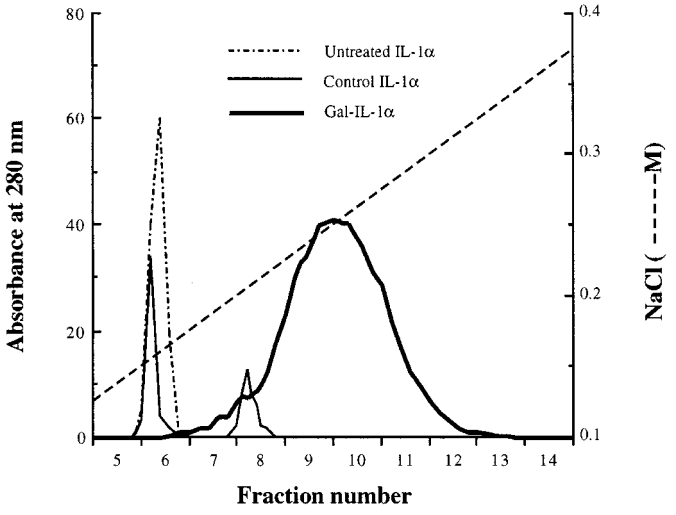


Figure 1. Purification of Gal-IL-1*a*. Untreated rhIL-1*a* (100 μg), control IL-1*a* (500 μg) or Gal-IL-1*a* (2 mg) after reaction were applied to the anion-exchange chromatography column (Mono Q, Pharmacia) with FPLC system. The starting buffer was 20 mM Tris-HCl (pH 7.0), and the elution buffer was the starting buffer containing 0.5 M NaCl with a linear gradient. Fractions (1 ml) were collected at a flow rate of 1 ml min^{-1}. For comparison the absorbance of untreated IL-1*a* was expanded to five fold.

Coupling of **3** with rhIL-1α

Compound **3** was transformed to an acyl azide derivative by reaction with hydrogen nitrite. The acyl azide derivative was coupled with rhIL-1α in 0.4 M sodium borate buffer (pH 10.0) for 60 min to afford a glycosylated rhIL-1α (Scheme 1). The rhIL-1 treated in the same manner without the acyl azide derivative was used as control (mock treated) IL-1α.

Purification of galactose-introduced IL-1α

The galactose bound IL-1 (Gal-IL-1α) was purified employing FPLC system using an anion-exchange chromatography column. As shown in Figure 1, untreated IL-1α eluted in Fr. 6, and treated IL-1α in Fr. 6 and 8. The peak at Fr. 8 of treated IL-1 seemed to be due to the conformational changes caused by the reaction condtion. In contrast, glycosylated IL-1 eluted in Fr. 7 to 12 peaking at Fr. 9 and 10. We then pooled Fr. 9 to 11 as glycosylated IL-1α. The altered elution profile of glycosylated IL-1 suggests that amino residues of IL-1 reacted with Gal-C9. In our preliminary analysis with TOF-MAS, the glycosylated IL-1α consists of three major products with 7 ± 1 mol Gal-C9 per molar IL-1, and uncoupled IL-1 was not detected (unpublished observations). This reflects the relatively broad elution profile of the glycosylated IL-1 on column chromatography.

SDS-PAGE analysis of Gal-IL-1α and lectin blotting

Untreated, treated and Gal-IL-1 were analysed on SDS-PAGE. As shown in Figure 2A, untreated IL-1 and treated IL-1 migrated at 18 kDa, and Gal-IL-1 at 22.4 kDa. The increased MW of the Gal-IL-1 represents the galactose introduction. Based on the difference of the MW, Gal introduce into IL-1 was estimated at 9.1 mol per molar IL-1. By the acyl azide method, the carbohydrate derivative theoretically reacts with amino residues. As rhIL-1α

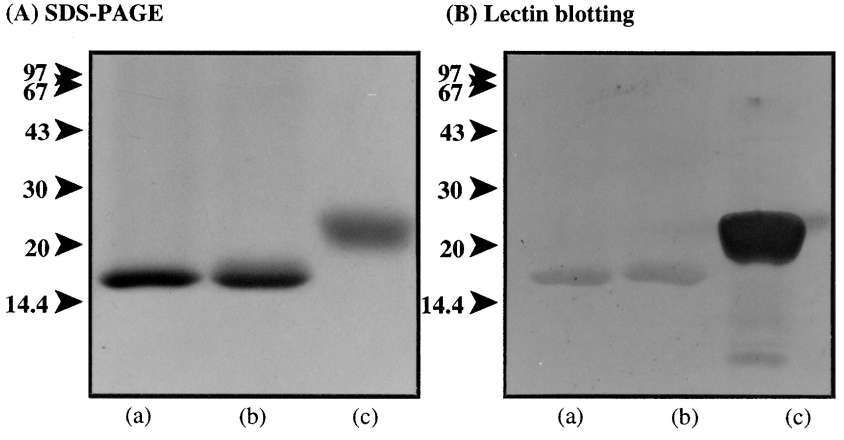


Figure 2. SDS-PAGE and lectin blot analysis of galactose-introduced IL-1*a*. (A) Fifteen microgram of IL-1*a*s were analysed on 15% polyacrylamide gel under reduction condition. The IL-1*a*s were visualized with Coomassie brilliant blue staining. (B) After electrophoresis, the IL-*a*s were transfered onto a PVDF membrane and blotted with HRP-conjugated RCA120. Molecular weight standard electrophoresed in parallel are indicated to the left in kilodaltons. (a) untreated IL-1*a* (b) control IL-1*a* (c) Gal-IL-1*a*

contains 33 amino residues per molecule, about one per 3.6 molar amino residues appeared to be reacted. To confirm that Gal was introduced into IL-1, lectin blot analysis was conducted using Gal-specific *R. communis* lectin (RCA120). As shown in Figure 2B, only Gal-IL-1 was markedly stained indicating that Gal was coupled to Gal-IL-1. The faint staining of untreated and control IL-1s was nonspecific because nonglycosylated molecular weight markers were also stained at the same density (data not shown).

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