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Pregnancy-associated Changes in Oligomannose Oligosaccharides of Human and Bovine Uromodulin (Tamm-Horsfall Glycoprotein)

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The urinary glycoprotein uromodulin (Tamm-Horsfall glycoprotein) exhibits a pregnancy-associated ability to inhibit antigen-specific T cell proliferation, and the activity is associated with a carbohydrate moiety [Muchmore and Decker (1985) Science 229:479-81; Hession *et al.*, (1987) Science 237:1479-84; Muchmore, Shifrin and Decker (1987) J Immunol 138:2547-53]. We report here that the $Man_{6(7)}GlcNAc_2$-R glycopeptides derived from uromodulin inhibit antigen-specific T cell proliferation by 50% at 0.2-2 μM, and further studies, reported elsewhere, confirm that oligomannose glycopeptides from other sources are also inhibitory, with $Man_9GlcNAc_2$-R the most inhibitory of those tested [Muchmore *et al.*, J Leukocyte Biol (in press)]. In this work, we have extended the observation of pregnancy-associated inhibitory activity to a second species, and have compared the oligomannose profile of Tamm-Horsfall glycoprotein (nonpregnant) with that of uromodulin (pregnant) derived from both human and bovine sources. Surprisingly, there was a pregnancy-associated decrease in the total content of oligomannose chains due predominantly to a reduction in $Man_5GlcNAc_2$-R and $Man_6GlcNAc_2$-R. $Man_7GlcNAc_2$-R, which did not decrease with pregnancy, comprised a significantly greater proportion of the total oligomannose chains in pregnant vs. nonpregnant samples from both species (human; 34.6% vs. 25.9%: bovine; 14.4% vs. 7.2%).

Uromodulin was originally isolated from human pregnancy urine based on its ability to inhibit antigen-specific T cell proliferation [1], and later the polypeptide was shown to be identical to that of Tamm-Horsfall (T-H) glycoprotein [2], which had been described in 1952 [3]. Since the inhibitory activity appeared to reside in a carbohydrate moiety [4], follow-up studies have focussed on 1) characterization of the inhibitory glycopeptide(s) and 2)

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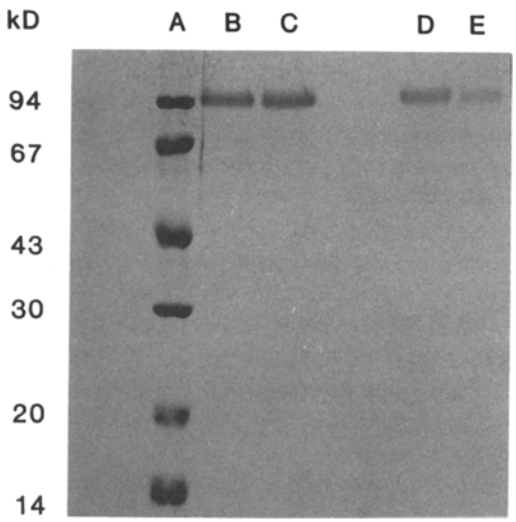


Figure 1. SDS PAGE of bovine T-H glycoprotein/uromodulin. T-H glycoprotein and uromodulin were prepared by salt precipitation of urine from nonpregnant or pregnant subjects, respectively; and SDS-PAGE was performed as described in the Materials and Methods section. A) standard mixture; B) human T-H glycoprotein (male); C) human uromodulin (female-pregnant); D) bovine T-H glycoprotein (female-nonpregnant); E) bovine uromodulin (female-pregnant).

comparison of the oligosaccharides of this urinary glycoprotein derived from pregnant (uromodulin) and nonpregnant (T-H glycoprotein) sources. Even though uromodulin is unlikely to play a role in systemic immunosuppression, since it is synthesized exclusively in the kidney [2] and low concentrations are found in the circulation [5], identification of an inhibitory oligosaccharide or glycopeptide could add significantly to our understanding of immunoregulation at the molecular level.

Antigen-specific T cell proliferation, utilized as the assay for inhibitory activity, requires interaction of antigen-presenting cells with T lymphocytes and is a critical component in a fully functional immune system. Antigen-specific T cell proliferation is strongly inhibited by the disaccharide Manα1-6Man [6] and to a lesser extent by specific monosaccharides [7]. This reaction is also inhibited by degradation products of yeast mannan [8, 9], an observation which may account for the pronounced immunosuppression exhibited by patients with chronic mucocutaneous candidiasis [10]. T-H glycoprotein, with an estimated 5 N-linked chains and no O-linked chains [11] per 615-amino acid polypeptide [2], contains a small proportion (1-20%) of oligomannose chains [11-13], although the predominant structures consist of tri- and tetra-antennary complex type oligosaccharides [14], which sometimes terminate in a unique β-linked *N*-acetylgalactosamine, or Sd^a antigen [15].

We report here that a glycopeptide preparation isolated from uromodulin containing 80% $Man_6GlcNAc_2$-R and 20% $Man_7GlcNAc_2$-R shows considerable inhibitory activity, suppressing antigen-specific T cell proliferation by 50% at concentration of 0.2-1 μM, and that the complex oligosaccharides derived from uromodulin are inactive. Subsequent to this

observation, several oligomannose glycopeptides were isolated from other sources and shown to be inhibitory [16]. $Man_9GlcNAc_2$-R was the most inhibitory of those tested [16]. Furthermore, ovalbumin $Man_6GlcNAc_2$-R, which contained approximately 8% hybrid contaminants, was approximately 50-fold less active than uromodulin $Man_6GlcNAc_2$-R, which contained 20% $Man_7GlcNAc_2$-R, even though the Man_6 structures were identical, as determined by NMR.

This suggested that the inhibitory activity of oligomannose oligosaccharides increased with chain length, and led us to propose that uromodulin may contain a higher proportion of long-chain (Man_7 and larger) oligomannose oligosaccharides than the corresponding T-H glycoprotein. Thus we have compared the oligomannose profile of human uromodulin with that of T-H glycoprotein, and have extended these studies to a second species, providing information on the inhibitory activity and oligomannose profile of bovine uromodulin and T-H glycoprotein. Our results show that the pregnancy-associated inhibition of antigen-specific T cell proliferation by the urinary glycoprotein uromodulin is not restricted to humans, but also occurs in Holstein dairy cows. Furthermore, glycosylation of both human and bovine uromodulin is enriched in Man_7 relative to T-H glycoprotein.

Materials and Methods

Preparation of T-H Glycoprotein and Uromodulin

Human urine was collected from individual female donors, aged 21-35, who were non-pregnant or pregnant (third trimester) at the time of collection. Bovine urine was collected from healthy 2-3 year old non-pregnant or pregnant (third trimester) Holstein dairy cows by catheterization. All urine samples were frozen within 2 h of collection. Urine samples were thawed, subjected to salt-precipitation as described by Tamm and Horsfall [3]; the precipitate was concentrated and dialyzed against distilled water with a collodion bag apparatus (Schleicher and Schuell, Keene, NH, USA). The product, which yields a single band on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1) is referred to as T-H glycoprotein when derived from a non-pregnant source and uromodulin when derived from a pregnant source.

SDS PAGE

Samples were heated with sample buffer, and electrophoresed on a 10% polyacrylamide gel according to the method of Laemmli [17]. Bands were visualized with Coomassie blue.

Antigen-specific T Cell Proliferative Assay

Human or bovine blood was collected by venipuncture, and peripheral blood lymphocytes were separated from autologous plasma and centrifuged on Ficoll-Hypaque. Since Holstein cows are not routinely immunized against tetanus, a single animal was immunized against tetanus and provided all the bovine lymphocytes used in this report. Antigen-specific T cell proliferation was measured by tetanus toxoid assay [1]. The recovered lymphocytes were washed three times in phosphate-buffered saline, pH 7.4, and 2-5 x 10^5 cells were plated in a total volume of 200 ml of RPMI 1640 medium containing 10% autologous plasma with

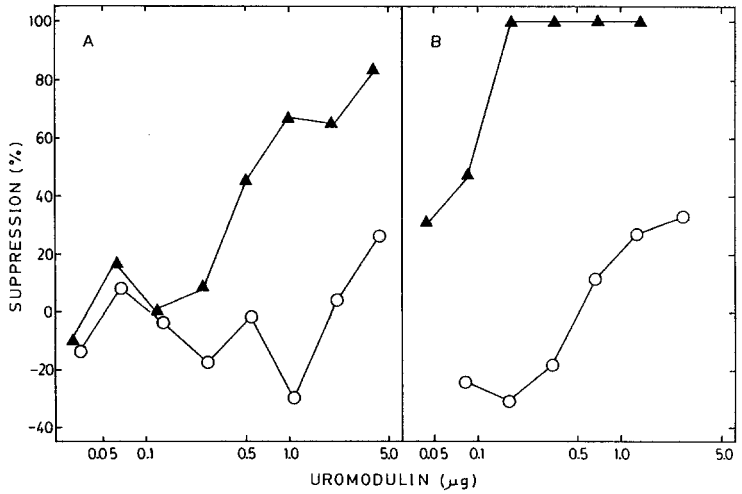


Figure 2. Suppression of antigen-specific T cell proliferation by bovine uromodulin. Human (panel A) or bovine (panel B) peripheral blood lymphocytes were stimulated with tetanus toxoid in the presence or absence of various amounts of glycoprotein. Bovine T-H glycoprotein (○); bovine uromodulin (▲). Suppression was calculated as 100% x [1.0 - (cpm_{ex} - cpm_m)/(cpm_t - cpm_m)] where the variables represent the cpm of incorporated ^{3}H-thymidine from cells incubated with media alone (cpm_m), media plus tetanus toxoid (cpm_t), or tetanus toxoid and uromodulin (cpm_{ex}).

or without 1 μl/well tetanus toxoid (Massachusetts Biologic Laboratories, Boston, MA, USA). Following incubation at 37°C for five days, cells were pulsed 16 h with 1 μCi/well ^{3}H-thymidine and harvested. Incorporation of radioactivity was determined by counting harvested cells in 3 ml Ecolume in a Beckman LS3801 scintillation counter. The inhibition of suppression (%) was calculated by 100 x [1 - (cpm+)/(cpm-)] where cpm+ represents the cpm in the presence of the sample and cpm- represents the cpm in the uninhibited control.

Isolation of Inhibitory Oligosaccharides from Human Uromodulin

A volume of 50-100 ml of uromodulin (0.3 mg/ml), prepared from pooled urine of human pregnant donors, was incubated with *Streptomyces griseus* Pronase (0.5 mg/ml) in 10 mM Tris, pH 7.5, 1 mM $MgCl_2$ with a drop of toluene for 16 h at 37°C. Following heat inactivation, the digest was lyophilized, resuspended in 5-15 ml of distilled water, and applied to a 2.5 x 85 cm column of Bio-Gel P-10 in 0.1 M pyridinium acetate, pH 6.0. Fractions (13 ml) were pooled and lyophilized to constitute fractions I-VII (see Fig. 3). Fraction IV was redigested with Pronase and rechromatographed on Bio-Gel P-10 to produce fraction IV-27, which retained the bulk of the inhibitory activity (see Fig. 4); fraction IV-27 was further treated with endoglycosidase H (16 h, 37°C, 50 mM sodium citrate, pH 6.0, 0.01 unit enzyme), desalted by ion-exchange chromatography, lyophilized, and separated by high performance liquid chromatography [18]. HPLC fraction IV-27-6 constituted the major peak, and retained the inhibitory activity (see Fig. 4). Structural information on IV-27 and IV-27-6 was obtained by ^{1}H-NMR as described below.

Analytical Methods

Protein was determined by the method of Lowry *et al.* [19], and sialic acid by a modification [20] of the thiobarbituric acid assay [21]. Neutral hexose was assayed by mixing 30 µl of sample with 150 µl 15 M sulfuric acid, heating at 100°C for 3 min, adding 30 µl 0.3% cysteine in distilled water, and comparing the A_{405} after 30 min with a standard curve produced by D-mannose. Total carbohydrate analysis of uromodulin and selected fractions was performed by gas-liquid chromatography of the trimethylsilyl derivatives following methanolysis [22, 23]. A methyl silicone column (25 mm i.d. x 25 m) was used for the separation, and inositol was the internal standard. Thin layer chromatography was performed on Whatman LHP-K high performance silica gel plates with a mobile phase of isobutanol/ethanol/water, 40/40/20 by vol, and saccharides were visualized with orcinol-ferric chloride spray (Sigma, St. Louis, MO, USA).

Glycopeptide and oligosaccharide samples for NMR analysis were repeatedly dissolved in ^2H_2O (99.96 atom% ^2H, Aldrich, Milwaukee, WI, USA) at room temperature and p^{2}H 6 with intermediate lyophilization. The deuterium-exchanged samples were subjected to ^{1}H-NMR spectroscopy at 500 MHz (Bruker AM-500 instrument interfaced with Aspect-3000 computer). Further experimental details have been described previously [24]. ^{1}H Chemical shifts are expressed in ppm downfield from internal 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). They were actually measured at 27°C relative to internal acetone (δ 2.225) with an accuracy of 0.002 ppm.

Analysis of Oligomannose Oligosaccharides

Uromodulin samples (0.1-1.0 mg protein) were lyophilized, taken up in 100 µl 10 mM Tris, pH 7.5, 0.01 M $MgCl_2$, and 5 µl Pronase (10 mg/ml) was added. Following incubation at 37°C for 16 h, the sample was heat inactivated 5 min in a boiling water bath, and 100 µl of endoglycosidase H (endo H, 0.01 unit, Genzyme, Boston, MA, USA) in 50 mM sodium citrate, pH 6.0, was added [25]. Following incubation at 37°C for 16 - 20 h, 630 µl ice-cold ethanol was added; the sample was centrifuged at 5,000 rpm for 10 min, and the supernatant saved; the pellet was washed with 75% ethanol and the supernatants pooled and evaporated to dryness under nitrogen [26]. Released oligosaccharides were labeled by incubation with 20 ml NaB^3H_4 (1.67 mCi/ml; sp. act. 600 mCi/mmol; NEN, Boston, MA, USA) in 0.3 ml 0.2 M sodium borate, pH 9.8 at 30°C for 4 h, followed by addition of 1 M acetic acid until the pH was below 4.6 [27].

Labeled oligosaccharides were separated from other glycopeptides by Con A affinity chromatography [28, 29] and then desalted on Bio-Gel P-2. The sample was applied to a 2 ml column of Con A agarose (Sigma C-7911) equilibrated with TBS (0.15 M NaCl, 0.01 M Tris, pH 8.0, 1 mM $CaCl_2$ and 1 mM $MgCl_2$), washed with 20 ml TBS, eluted with 20 ml of 10 mM α-methyl glucoside in TBS, and then with 35 ml 0.1 M α-methyl mannoside in TBS at 65-70°C. Fractions eluting with the α-methyl mannoside were pooled, lyophilized, taken up in 1-1.5 ml distilled water and applied to a 150 ml column of Bio-Gel P-2 equilibrated with distilled water. Fractions of 5 ml were collected, and 0.3 ml aliquots were taken for scintillation counting. Fractions having greater than 100 cpm were pooled and lyophilized.

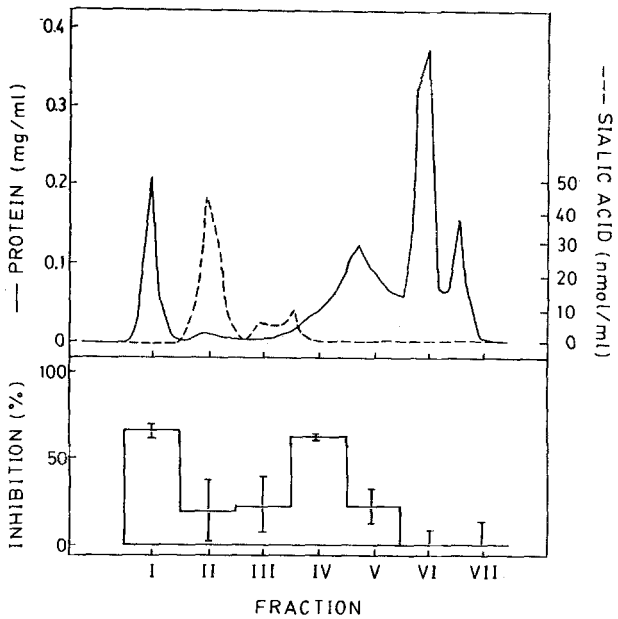


Figure 3. Suppressive activity of uromodulin fractions following Pronase digestion and Bio-Gel P-10 chromatography. Uromodulin (15 mg) was digested with Pronase and the digest was fractionated on Bio-Gel P-10 as described in the Materials and Methods section. Following analysis of individual fractions (upper panel), fractions were pooled, lyophilized, reconstituted in 0.6 ml distilled water, and 2 µl were added and serially diluted to determine % inhibition in the antigen-specific proliferative assay as a function of concentration. Data shown (lower panel) correspond to the addition of 0.012 µl of each fraction.

Analysis of the labeled oligosaccharides was performed by high performance liquid chromatography on a Waters Glyco-Pak N column with a mobile phase of acetonitrile/H_2O, 66/34 by vol, and a flow rate of 1 ml/min. Calibration was performed by chromatographing 10-20 µg of a standard mixture of reduced oligomannose chains (MSE, Genzyme), and by monitoring the absorbance at a wavelength of 195 nm (Waters Model 480 detector) with output on a Hewlett Packard 3390A integrator. Radiolabeled samples were collected into mini scintillation vials using a Gilson Model FC-100 fraction collector (0.5 or 1 min fractions), 5 ml Ecolume (ICN, Costa Mesa, CA, USA) was added, and samples were counted for 5 min in a Beckman 3801 scintillation counter. In order to determine the calibration necessary to align the radioactive profile with the chromatogram, a sample of ^{3}H-leucine containing 10 µg and 200,000 dpm was chromatographed.

Results

Isolation of Bovine and Human Uromodulin

Utilizing the salt precipitation method of Tamm and Horsfall [3], a preparation containing a single diffuse band by SDS-polyacrylamide gel electrophoresis can readily be obtained from human or bovine urine (Fig. 1). The sizes of the glycoproteins derived from human and

bovine urine are clearly similar, with apparent molecular weights of 97,000 and 99,000 for the human and bovine glycoproteins, respectively. Since the glycoprotein migrates as such a diffuse band, no pregnancy-associated differences in the migration are observed under these conditions. Human male T-H glycoprotein, which is inactive in the tetanus toxoid assay, is indistinguishable from the glycoprotein derived from pregnant (Fig. 1) or nonpregnant (data not shown) females. Although the yield of isolated glycoprotein is extremely variable, the average recovery of glycoprotein from bovine urine is less than that from human urine (5 mg protein/l vs. 20 mg protein/l). The glycoprotein isolated from bovine urine reacts with antibody to human T-H glycoprotein (uromucoid) as determined by enzyme-linked immunosorbent assay (data not shown).

Inhibition of Antigen-specific T Cell Proliferation

Previously uromodulin, isolated from human pregnancy urine, was shown to inhibit the proliferation of peripheral blood lymphocytes stimulated by tetanus toxoid [1, 2]. Fig. 2 shows that bovine uromodulin effectively inhibits antigen-specific proliferation of human or bovine T cells, whereas bovine T-H glycoprotein is inhibitory only at the highest doses. Based on dose-response curves similar to Fig. 2, we estimated the ID_{50} (the amount of protein required to suppress the assay by 50%) for several samples. The average ID_{50} was significantly higher ($p<0.025$) for bovine T-H glycoprotein (0.82±0.48 mg protein, N = 8) than for bovine uromodulin (0.28±0.30 mg protein, N = 5). This is similar to the difference observed between human T-H glycoprotein and human uromodulin, for which the ID_{50} values in a human assay were >1.0 and 0.13 mg protein, respectively [2]. Thus the pregnancy-associated increase in inhibitory activity, previously described for human uromodulin, also occurs in Holstein cows.

Isolation of an Oligosaccharide which Inhibits Antigen-specific Proliferation

Following Pronase treatment of human uromodulin, inhibitory activity was recovered in a fraction which had the characteristics of an oligomannose glycopeptide (Fig. 3, fraction IV). The additional inhibitory activity associated with the void volume (Fig. 3, fraction I) was attributed to incomplete digestion of uromodulin. Fraction IV had a mannose:*N*-acetylglucosamine ratio of 3.4:1 and accounted for 20% of the mannose recovered from the column; the remainder of the recovered mannose was associated with fraction II, which had a composition consistent with that of the dominant tri- and tetra-antennary chains which have previously been described for human T-H glycoprotein [14].

Treatment of fraction IV with either endoglycosidase F or endoglycosidase H resulted in complete digestion as monitored by the increased mobility of the saccharide moiety on thin layer chromatography (data not shown). Samples digested with endoglycosidase F or endoglycosidase H showed inhibitory activity comparable to the incubated control (data not shown), suggesting that an intact carbohydrate-peptide linkage is not essential for inhibition.

In order to prepare a sample of fraction IV suitable for structural characterization by NMR, fraction IV was redigested overnight with Pronase and rechromatographed on Bio-Gel P-10. Fraction IV-27, which comprised the bulk of the carbohydrate recovered from the column, retained most of the inhibitory activity (Fig. 4) and was selected for structural analysis.

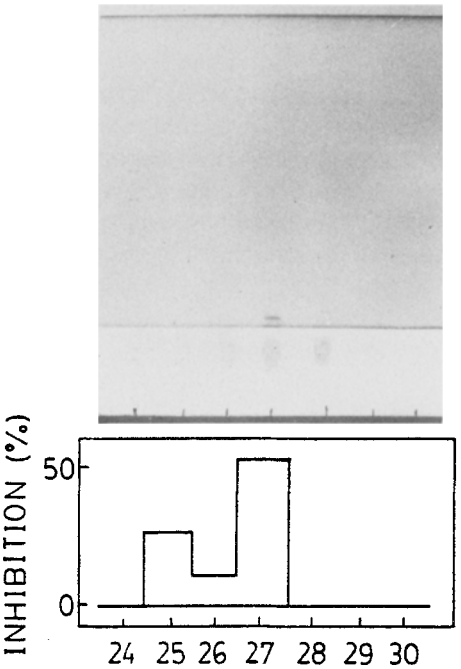


Figure 4. Analysis of fractions following redigestion of fraction IV (Fig. 3) with Pronase and chromatography on Bio-Gel P-10. Fractions were lyophilized and reconstituted with 0.5 ml distilled water. Upper panel: thin layer chromatography of 2 µl of each fraction; lower panel: inhibition of tetanus toxoid assay by addition of 0.11 µl of each fraction.

The active fraction IV-27 was lyophilized and analyzed by 500-MHz ^{1}H-NMR spectroscopy (Table 1). The characterization of the structures of the carbohydrate moieties was accomplished by comparison of the chemical shifts of the mannose and *N*-acetylglucosamine anomeric protons (H-1's), mannose C-2 protons (H-2's), and *N*-acetylglucosamine *N*-acetyl methyl protons (NAc's) for the uromodulin glycopeptides to those reported for a large number of oligomannose N-type glycopeptides and oligosaccharides [24, 30-34].

The ^{1}H-NMR spectrum for fraction IV-27 is consistent with that of a mixture of two oligomannose glycopeptides [$Man_6GlcNAc_2$-R (Man_6)and $Man_7GlcNAc_2$-R (Man_7)] which differ from each other in the presence of a terminal α(1-2)-linked mannosyl residue (D_1, Fig. 5). The two components have in common the double-3,6-branched $Man_5GlcNAc_2Asn$ core moiety typical of oligomannose N-type oligosaccharides (residues A, B, C, 4, 4', 3, 2, and 1, Fig. 5). This element is characterized by the chemical shifts of the anomeric-proton signals of Man-4, Man-4', terminal Man-A and terminal Man-B (see Table 1); these resonances are observed in the intensity ratio of 1:1:1:1. The difference between the two components in the uromodulin sample is restricted to the Man-4 branch. While the major component ($Man_6GlcNAc_2$-R) has its Man-4 extended by one mannose residue in α(1-2)-linkage (Man-C), the minor component ($Man_7GlcNAc_2$-R) has another mannose α(1-2)-attached to Man-

Table 1. ^1H Chemical shifts of pertinent structural-reporter groups of monosaccharides present in the immunosuppressive glycopeptides and oligosaccharides derived from uromodulin, as compared to oligomannose carbohydrates obtained from other glycoproteins.

Reporter group	Residue[a]	Chemical shift[b] in					
		glycopeptides				oligosaccharides	
		uromodulin		*ovalbumin*		*uromodulin*	
		Man_6-$GlcNAc_2Asn$	Man_7-$GlcNAc_2Asn$	Man_5-$GlcNAc_2Asn$	Man_6-$GlcNAc_2Asn$	Man_6-GlcNAc	Man_7-GlcNAc
H-1	GlcNAc-1	5.07	5.07	5.071	5.073	--	--
	GlcNAc-2	4.604	4.604	4.606	4.607	--	--
	GlcNAc-2α	--	--	--	--	5.247	5.245
	GlcNAc-2β	--	--	--	--	n.d.[c]	n.d.[c]
	Man-3	n.d.[c]	n.d.[c]	4.781	4.774	n.d.[c]	n.d.[c]
	Man-4	5.349	5.342	5.099	5.350	5.351	5.345
	Man-C	5.052	5.305	--	5.055	5.055	5.305
	Man-D_1	--	5.052	--	--	--	5.052
	Man-4′	4.871	4.871	4.872	4.875	4.874	4.874
	Man-A	5.095	5.095	5.093	5.097	--	--
	Man-A(α)	--	--	--	--	5.083	5.081
	Man-A(β)	--	--	--	--	5.109	5.108
	Man-D_2	--	--	--	--	--	--
	Man-B	4.908	4.908	4.908	4.912	4.910	4.910
	Man-D_3	--	--	--	--	--	--
H-2	Man-3	4.232	4.232	4.251	4.235	--	--
	Man-3(α)	--	--	--	--	4.246	4.244
	Man-3(β)	--	--	--	--	4.234	4.232
	Man-4	4.114	4.114	4.077	4.117	4.118	4.117
	Man-C	4.067	4.105	--	4.070	4.069	4.110
	Man-D_1	--	4.067	--	--	--	4.068
	Man-4′	4.144	4.144	4.144	4.147	4.144	4.144
	Man-A	4.067	4.067	4.066	4.070	4.069	4.068
	Man-D_2	--	--	--	--	--	--
	Man-B	3.983	3.983	3.985	3.990	3.991	3.992
	Man-D_3	--	--	--	--	--	--
NAc	GlcNAc-1	2.014	2.014	2.012	2.016	--	--
	GlcNAc-2	2.062	2.062	2.060	2.064	--	--
	GlcNAc-2α,β	--	--	--	--	2.043	2.042

[a] For coding of monosaccharide residues, see Fig. 5. In this column, α and β refer to the respective anomeric forms of the reducing oligosaccharide.

[b] The NMR spectra were acquired at 500 MHz by examining neutral ^2H_2O solutions of the compounds at 27°C.

[c] n.d., the value could not be determined, as the resonance was obscured by the residual H^2HO signal.

C (denoted as Man-D_1). The chemical shift increments typical of the presence of Man-D_1 are observed: for H-1 of Man-C, from δ 5.05 to 5.30, and for H-1 of Man-4, from δ5.35 to 5.34 The ratio of Man_6 and Man_7 in the mixture was deduced to be 4:1, from the intensity ratio of the H-1 signals of substituted Man-C (δ 5.305) and Man-4 (δ ~5.345), being 1:5.

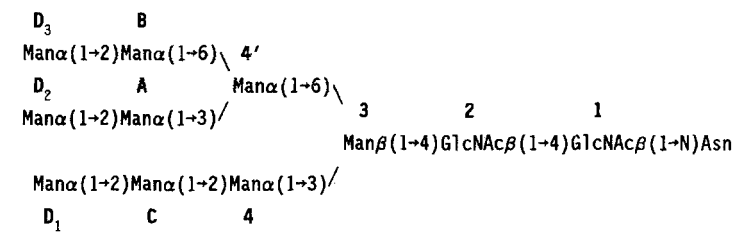


Figure 5. Coding system commonly used for the monosaccharide residues in oligomannose oligosaccharide chains.

Following NMR analysis of fraction IV-27, this fraction was treated with endoglycosidase H and fractionated by HPLC [18]. The major fraction (IV-27-6) was also subjected to NMR analysis and tested for inhibitory activity. Fig. 6 shows that this oligosaccharide preparation is still inhibitory, whereas other HPLC fractions, tested as controls, were not inhibitory (controls not shown). By NMR analysis, this sample appears to be the endo H cleavage product for the Man_6 glycopeptide described above (>95%), containing up to 5% of the Man_7 oligosaccharide. Although the limited quantities of fractions IV-27 and IV-27-6 make a definitive assessment of their relative inhibitory activities difficult, the data suggest that the ID_{50} for IV-27-6 is approximately 1 μM, whereas both IV-27 and IV effectively achieve 50% inhibition at concentrations lower than 1 μM. Thus the oligosaccharide preparation, which is greater than 95% $Man_6GlcNAc$, may be less active than the 80/20 Man_6/Man_7 glycopeptide preparation from which it was derived.

Analysis of Oligomannose Oligosaccharides

Following the characterization of an oligomannose oligosaccharide preparation with inhibitory activity, we further demonstrated that oligomannose oligosaccharides from sources other than uromodulin also have inhibitory activity, which appears to increase with chain length [16]. We proposed that there are pregnancy-associated changes in the glycosylation of uromodulin, leading to an alteration in the profile and/or content of oligomannose chains.

To test this hypothesis, oligomannose chains were released from uromodulin or T-H glycoprotein by sequential treatment with Pronase and endo H, labeled with NaB^3H_4, isolated by Con A affinity chromatography, and desalted by Bio-Gel P-2 chromatography prior to analysis by HPLC. This experimental approach was largely dictated by technical considerations. Several preliminary studies revealed that the oligomannose oligosaccharides were not effectively released from intact denatured bovine T-H glycoprotein by either N-glycanase or endoglycosidase H. Pre-treatment of the glycoprotein with Pronase enhanced the release of oligomannose chains with endo H, but limited the usefulness of N-glycanase. Thus we chose to focus on the oligomannose chains, because a full characterization of all N-linked chains released by N-glycanase was not technically feasible. Following Pronase and endo H treatment, and labeling with NaB^3H_4, oligomannose oligosaccharides were readily separated from glycopeptides utilizing Con A affinity chromatography. Alternative methods, such as ion exchange chromatography, were inefficient in removing ^3H-labeled contaminants.

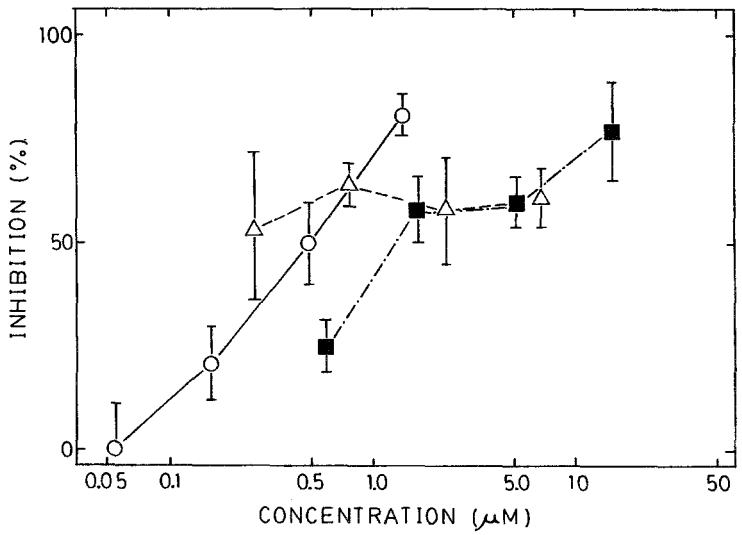


Figure 6. Inhibition of antigen-specific proliferation by glycopeptides and purified $Man_6GlcNAc$ from uromodulin. The concentration was estimated by dividing the mannose concentration (determined by gas-liquid chromatography or cysteine sulfuric acid assay) by 6. (○) fraction IV (Fig. 1); (△) fraction IV-27 (Fig. 8, NMR analysis in Table 1); (■) fraction IV-27-6 obtained by digestion of fraction IV-27 with endo H and separation by HPLC (NMR analysis in Table 1).

A representative HPLC profile of oligomannose oligosaccharides from human uromodulin is shown in Fig. 7. The dominant oligosaccharides, consistent with previous reports of T-H glycoprotein [12, 13], are $Man_5GlcNAc$, $Man_6GlcNAc$, and $Man_7GlcNAc$. Oligomannose chains could be reproducibly quantified from these HPLC profiles similar to Fig. 7, since duplicate analyses of a single sample differed by no more than 5% in the relative proportion of each component and by no more than 20% in total recovery.

The results indicate that the relative content of Man_7 oligosaccharides in human or bovine uromodulin is significantly higher than the corresponding T-H glycoprotein (Table 2). In the case of human T-H glycoprotein, where Man_7 already comprises 25% of the total, the pregnancy-associated increase in the relative content of Man_7 is modest. For bovine T-H glycoprotein, which initially contains little Man_7, pregnancy is associated with a doubling of the relative amount of Man_7. Although the relative amount of Man_7 increases in both cases, the *total* amount of oligomannose chains recovered appears to *decrease* during pregnancy. Thus the fairest presentation of the data is that the apparent increase in the relative amount of Man_7 is actually due to a net decrease in the amounts of Man_5 and Man_6.

The observation that the total content of oligomannose chains decreases during pregnancy, although surprising, was corroborated for the bovine samples by carbohydrate analysis. The carbohydrate composition of bovine T-H glycoprotein (Table 3) is similar to that reported previously for calf T-H glycoprotein, prepared by a modified salt precipitation method [35]. The ratio of mannose to galactose is significantly lower for uromodulin, prepared from pregnant donors, than for T-H glycoprotein, prepared from non-pregnant donors.

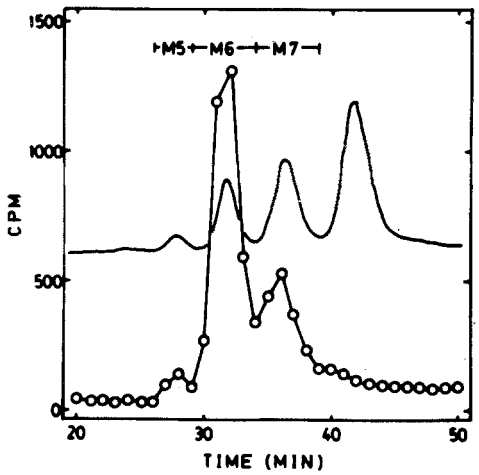
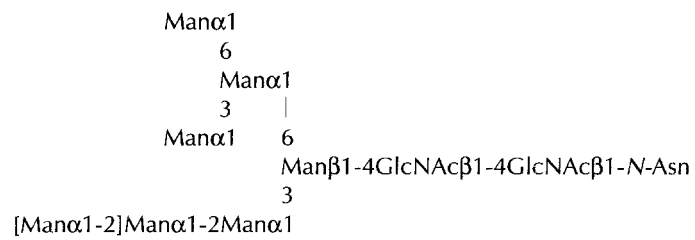


Figure 7. HPLC of oligomannose oligosaccharides from bovine T-H glycoprotein and uromodulin. Details of release, labeling, and purification of oligosaccharides and chromatographic conditions are given in the Materials and Methods section. The solid line represents A_{195} (0.02 absorbance units full-scale) for 20 μg of a standard mixture (Genzyme) containing Man_5, Man_6, Man_7, and Man_8.

Discussion

Although T-H glycoprotein has been isolated from a number of different species including human [3], rabbit [36], hamster [37], and bovine [35], no reports of the inhibition of antigen-specific T cell proliferation by uromodulin (pregnancy T-H glycoprotein) in species other than human have appeared. We report here that the pregnancy-associated changes in the inhibitory activity of T-H glycoprotein are not restricted to humans, but also occur in Holstein cows. This work additionally adds to the identification of the inhibitory component through 1) isolation of an inhibitory glycopeptide having the structure



and 2) demonstration of pregnancy-associated alterations in the oligomannose profile of human and bovine uromodulin. In the discussion below, we have focused first on the glycosylation changes followed by a discussion of the inhibitory activity of oligomannose oligosaccharides.

Table 2. Relative amounts of Man_5, Man_6, and Man_7 in the oligomannose oligosaccharides of uromodulin and T-H glycoprotein.

Sample	Recovered oligomannose oligosaccharides (%)			Total (cpm/mg protein)
	Man_5	Man_6	Man_7	
Human				
T-H glycoprotein (N=5)	7.7±1.5	66.4±2.0	25.9±1.5	76,000±34,000
Uromodulin (N=4)	6.2±2.4	59.2±6.2	34.6±5.0[a]	47,000±24,000
Bovine				
T-H glycoprotein (N=5)	24.9±2.9	67.9±3.0	7.2±2.5	21,000±9,000
Uromodulin (N=5)	23.9±3.2	61.8±5.0	14.4±4.7[a]	7,500±5,400[a]

[a]Mean differs from that of non-pregnant samples (by t-test, $p<0.025$).

Comparison of the oligomannose oligosaccharides of bovine T-H glycoprotein with those of bovine uromodulin reveal a dramatic reduction (63%) in total oligomannose chains associated with pregnancy. This reduction can be attributed to lower amounts of both Man_5 and Man_6 resulting in a pregnancy profile which is enriched in Man_7 relative to the nonpregnant condition. Similar, yet less dramatic, changes occur in human uromodulin relative to T-H glycoprotein.

Several other reports have focused on changes in glycosylation of a single glycoprotein associated with various conditions. As examples, a pregnancy-associated shift from all bi-antennary to ~10% tri-antennary chains was reported for human transcortin, isolated from serum [38]. Changes in the glycosylation of human α_1-acid glycoprotein [39] and IgG [40] have also been noted for clinical conditions such as cancer and rheumatoid arthritis. These serum glycoproteins typically do not contain oligomannose oligosaccharides, whereas glycoproteins synthesized by the kidney can often contain a mixture of oligomannose and complex tri- and tetra-antennary chains [41, 42]. Oligomannose chains are observed to decrease following transformation of baby hamster kidney cells with polyoma, with an increase in tetra-antennary chains [41]. Oligomannose chains of fibronectin also decrease with an increase in complex chains following treatment of chondrocytes with retinoic acid [43]. Thus although changes in the content of oligomannose chains have been demonstrated for various conditions, no previous work has demonstrated shifts in the profile of oligomannose chains.

Regarding the relationship of oligomannose composition to inhibitory activity of this urinary glycoprotein, we propose that the *relative* amounts of specific oligomannose chains may be more critical that the *absolute* amount of any particular oligosaccharide. Support for this hypothesis was obtained by plotting the ratio of Man_5/Man_7 vs. the ID_{50} for bovine uromodulin and T-H glycoprotein (Fig. 8). The results indicate a correlation between this ratio and the amount of protein required to suppress the assay by 50%, with a correlation coefficient of 0.85. Thus the critical factor in predicting inhibitory activity may not be the content of Man_7 (since bovine uromodulin has the same Man_7 content as bovine T-H glycoprotein), but rather

Table 3. Carbohydrate composition of bovine T-H glycoprotein and uromodulin as determined by gas liquid chromatography. Duplicate analyses of samples from three individuals in each group were performed.

Component	Mole/mole galactose		
	T-H Glycoprotein	Uromodulin	Calf[a]
Fuc	0.08±0.04	0.08±0.01	0.19
Man	0.96±0.05	0.72±0.13[b]	0.96
Gal	1.00	1.00	1.00
GalNAc	0.20±0.04	0.42±0.04	0.29
GlcNAc	2.00±0.26	2.15±0.76	1.53
Sialic acid	0.73±0.12	0.81±0.19	0.61

[a] Taken from van Dijk *et al.* [35].
[b] The mean is significantly different from that for the nonpregnant group as determined by a pooled t-test ($p<0.025$).

the ratio of Man_5 to Man_7. A similar analysis for human uromodulin also indicates that the activity and the Man_5/Man_7 ratio are inversely correlated; however, too few samples were analyzed to determine whether this trend was significant.

The potential that a specific profile of oligomannose chains may inhibit antigen-specific T cell proliferation is of interest from an immunoregulatory viewpoint. It is unlikely that uromodulin *per se* plays a role in immunosuppression, since it is synthesized in the kidney [2] and is not typically found in high concentrations in the circulation [5]. Few circulating glycoproteins are known to contain oligomannose oligosaccharides, notably IgD [44] and IgM [45], as a hepatic clearance system for these saccharides has been demonstrated [46] and circulating mannose-binding proteins have also been identified [47]. Conditions which lead to elevated levels of circulating oligomannose glycoproteins are predicted to result in pronounced immunosuppression. This has been documented in the case of chronic mucocutaneous candidiasis [10], and may potentially contribute to the immunosuppression which occurs during viral infection, as some viral glycoproteins are rich in oligomannose oligosaccharides [48]. Future studies aimed at the mechanism of suppression may elucidate a key immunoregulatory function for specific oligomannose oligosaccharides.

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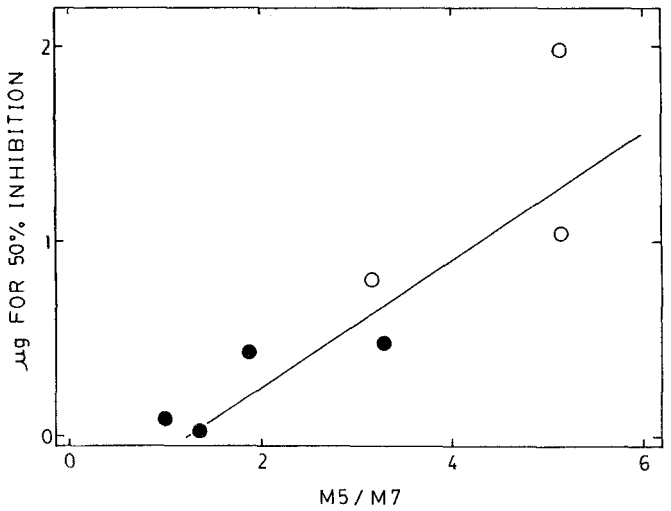

Figure 8. Comparison of the amount of bovine T-H glycoprotein/uromodulin required to suppress antigen-specific T cell proliferation by 50% (ordinate) with the ratio of Man_5 to Man_7 (abcissa). T-H Glycoprotein (nonpregnant, open circles); uromodulin (pregnant, closed circles). The correlation coefficient is 0.85.

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