Carbohydrate Composition and Identification of Blood Group A, B, and H Oligosaccharide Structures on Human Factor VIII/Von Willebrand Factor*

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Human Factor VIII/von Willebrand Factor purified from pooled normal plasma contains 3.7% sialic acid, 2.4% galactose, 4.0% N-acetylgalacosamine, 3.2% mannose, 1.0% fucose, and 0.7% N-acetylgalactosamine. By hemagglutination inhibition, it was demonstrated that type A, B, and O(H) blood group activities are associated with this glycoprotein. Type A blood group activity of both Factor VIII/von Willebrand Factor and asialo-porcine submaxillary mucin was significantly reduced by incubation with the limpet α-N-acetylgalactosaminidase which removes terminal GalNAc from A blood group structures. Complete activity was restored by reincorporation of GalNAc using the β-(α1 → 2 fucosyl)galactoside α1 → 3N-acetylgalactosaminyltransferase specific for H blood group acceptors. By measurement of the amount of [14C]GalNAc incorporated into Factor VIII/von Willebrand Factor before and after N-acetylgalactosaminidase treatment, the content of type A and II blood group structures was determined to be 0.7 ± 0.4 and 1.4 ± 0.8 mol/mol of 200,000 molecular weight subunit, respectively. Covalent association of these structures with the protein was confirmed by both lipid extraction and polyacrylamide gel experiments using N-acetylgalactosaminidase-treated Factor VIII/von Willebrand Factor which had [14C]GalNAc reincorporated into it. Treatment of this derivative with partially purified Streptococcus pneumoniae endo-β-galactosidase released a labeled trisaccharide identified as [14C]GalNAcα1 → 3(Fucα1 → 2)Gal. This same trisaccharide was released by endosidase treatment of the [14C]GalNAc derivative of fucosylated asialo-α1-acid glycoprotein containing terminal [14C]GalNAcα1 → 3(Fucα1 → 2)Galβ1 → 4GlNAc-R and a similarly labeled derivative of fucosylactose, [14C]GalNAcα1 → 3(Fucα1 → 2)Galβ1 → 4Galc. In contrast, labeled asialoglycoprotein submaxillary mucin containing α[14C]GalNAcα1 → 3(Fucα1 → 2)Galβ1 → 3GalNAc-R terminal sequence was resistant to endosidase digestion. It is concluded that terminal oligosaccharide sequences of GalNAcα1 → 3(Fucα1 → 2)Gal and Fucα1 → 2Gal corresponding to type A and H blood group active structures, respectively, are present on normal Factor VIII/von Willebrand protein in a β1 → 4 linkage to the penultimate sugar residue. Moreover, β-elimination experiments suggest that oligosaccharides containing these structures exist in an alkaline stable linkage to the protein. The observations reported here may bear on the recognized tendency for some hemophilic patients to develop antibodies toward FVIII/vWF following repeated infusions of FVIII/vWF pooled concentrates.

Human Factor VIII/von Willebrand Factor is isolated from plasma as a glycoprotein with two distinct but associated biological activities. These consist of a procoagulant activity which is characteristically low in classic hemophilia (1) and a platelet-aggregating activity which is frequently deficient in individuals with another bleeding disorder, von Willebrand’s disease (2, 3). In the latter disease, circulating FVIII/vWF protein levels may also be decreased, but often to a lesser extent than platelet aggregating activity. Recent reports have suggested that FVIII/vWF isolated from patients with von Willebrand’s disease may contain an abnormally low carbohydrate content as evidenced by decreased sialic acid values and negative carbohydrate stains on polyacrylamide gel (4, 5). The possibility that this disease may be related to an abnormality in the carbohydrate portion of FVIII/vWF is also supported by studies of the normal protein. Removal of sialic acid residues from normal FVIII/vWF promotes its rapid clearance from the circulation and decreases platelet-aggregating activity to 38% of normal (6). Further removal of terminal galactose residues from asialo-FVIII/vWF lowers platelet aggregating activity to 12% while 100% of the procoagulant activity is retained (7, 8). These observations were essentially confirmed by the finding that treatment of asialo-FVIII/vWF with galactose oxidase reduces platelet aggregating activity to 10% while subsequent reduction by potassium borohydride restores activity to near normal (9).

In essence the sum of all of these reports suggests that the carbohydrate moiety of FVIII/vWF is important for both optimal expression of platelet-aggregating activity and in vivo survival.

This report presents further progress on the characterization of the carbohydrate on FVIII/vWF, undertaken to establish

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lish a basis of comparison between normal and variant forms of FVIII/vWF. Of particular significance is the observation that purified FVIII/vWF possesses A, B, and H blood group activities, a potential concern since FVIII/vWF concentrations prepared from pooled plasma are used therapeutically. Evidence is provided to establish that oligosaccharide structures characteristic of blood group active substances are covalently linked to the FVIII/vWF molecule. A preliminary report of these results has been published (7).

**EXPERIMENTAL PROCEDURES**

**Materials**—Intermediate purity human FVIII/vWF concentrates purified from pooled plasma (10) were obtained from the American National Red Cross, Bethesda Md. FVIII/vWF was further purified to homogeneity as previously described (6), d-[1-14C]Galactosamine-HCl, D-[1-14C]Glucosamine-HCl, UDP-[1-14C]N-acetylglucosamine, CMP-[1H]NeuAc, and Protosol were products of New England New 2. Unlabeled UDP-GalNAc was prepared as previously described (11). 2-Fucosyllactose (12) was purified from human milk and all other sugars were purchased from Pfanzehl. Human antisera to A and B blood group substances were purchased from Hyland; purified avian red blood cells (type 1, Ulex europeaus) was obtained from P & L Biochemicals Inc; sugar determinations were a gift of Dr. Karl Schmid, Boston University. Porcine submaxillary mucin from pooled glands was purified and desialylated as described elsewhere (11).

The following enzymes were purified using published procedures: α-N-acetylgalactosaminidase from limpet (Patella vulgata) acetone powder (13); neuraminidase from Clostridium perfringens (14); β-(Gal→1→2 fucosyllactose α1→3 N-acetylglucosaminyltransferase from porcine submaxillary glands (11); and β-galactosidase α→1→6 sialyltransferase from bovine colostrum (15). Endo-β-galactosidase was obtained from the culture filtrate of Streptococcus pneumoniae by ammonium sulfate precipitation and gel filtration on Sephadex G-200 (equifrin) equilibrated in 25 mM sodium cacodylate, pH 6.5; it was used without further purification (16). All enzymatic reactions in this report are expressed as micromoles of product formed per min of incubation at 37°C.

**Carbohydrate Analysis**—Total sialic acid was determined using the thioharbituric acid assay (17) after releasing glycosidically bound sialic acid enzymatically with C. perfringens neuraminidase or by acid hydrolysis (6). Fucose was quantitated by the Diache-Schlett spectrum in human milk and all other sugars were purchased from Pfanzehl. Human antisera to A and B blood group substances were purchased from Hyland; purified avian red blood cells (type 1, Ulex europeaus) was obtained from P & L Biochemicals Inc; sugar determinations were a gift of Dr. Karl Schmid, Boston University. Porcine submaxillary mucin from pooled glands was purified and desialylated as described elsewhere (11).

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**RESULTS**

Carbohydrate Composition of FVIII/vWF—FVIII/vWF contains about 15% carbohydrate by weight consisting of sialic acid, galactose, N-acetylglucosamine, mannose, fucose, and N-acetylgalactosamine as listed in Table I. These sugars are common to glycoproteins which contain both asparagine-linked and threonine/serine-linked oligosaccharides (24).

**Demonstration of A, B, and H Blood Group Activity—**

[Note: The rest of the text is not provided here but would continue with further experimental details and results.]
agglutination of type A erythrocytes by anti-A sera was 0.8 μg. After treatment of the FVIII/vWF with α-N-acetylgalactosaminidase, which removes the terminal GalNAc from A blood group structures to give H blood group structure, no hemagglutination inhibition could be detected at the highest levels tested (50 μg). Inhibition of hemagglutination could be restored to normal levels by incubation with UDP-[14C]-GalNAc and N-acetylgalactosaminyltransferase which converts H blood group structures to A. Moreover, after treatment with N-acetylgalactosaminyltransferase, a small increase in the ability of native FVIII/vWF to inhibit hemagglutination by anti-A sera was also observed which suggests a conversion of intrinsic H oligosaccharides to those of A blood group structure (Table II). Similar results were obtained with porcine submaxillary mucin which contains both A and H blood groups (25) and was used as a control protein.

Since the N-acetylgalactosaminyltransferase has a strict acceptor specificity for the II blood group structure, Fucα1 → 2Gal-R, the amount of [14C]GalNAc incorporated under saturating conditions is equivalent to the number of intrinsic H oligosaccharides present in the native FVIII/vWF protein prepared from pooled plasma. The content of A blood group oligosaccharides may be estimated by similarly measuring the increase in H blood group structures produced after treatment of the FVIII/vWF with N-acetylgalactosaminidase. Thus, by a comparison of the incorporation of [14C]GalNAc into the native and N-acetylgalactosaminidase-treated glycoproteins, an estimation of the II and A blood group sites can be obtained. Analysis of three different preparations of native and N-acetylgalactosaminidase-treated FVIII/vWF indicated average values for the H and A blood group oligosaccharides of 1.4 ± 0.8 and 0.7 ± 0.4 mol/mol of Mγ = 200,000 subunit, respectively. The content of II and A blood group structures in asialo-porcine submaxillary mucin from pooled glands was determined to be 0.56 and 0.12 nmol/mg, respectively, in good agreement with earlier structural studies (25).

**Covalent Association of Blood Group Active Oligosaccharides with FVIII/vWF**—To exclude the possibility that blood group active oligosaccharides of FVIII/vWF were due to adsorbed glycolipids, FVIII/vWF was extracted with chloroform:methanol and the resulting fractions analyzed for their content of amino sugars which are common to blood group active glycolipids. The protein fraction recovered after extraction contained 170 ± 17 nmol/mg of N-acetylglucosamine and

**Carbohydrate on Human Factor VIII/von Willebrand Protein**

<table>
<thead>
<tr>
<th>Structure</th>
<th>Blood Group Activity</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Gal1-R</td>
</tr>
<tr>
<td>2</td>
<td>GalNAc1-3Gal1-R</td>
</tr>
<tr>
<td>3</td>
<td>Gal1-3Gal1-R</td>
</tr>
<tr>
<td>4</td>
<td>[14C]GalNAc1-3Gal1-4Glc</td>
</tr>
<tr>
<td>5</td>
<td>[14C]GalNAc1-3Gal1-4GlcNAc</td>
</tr>
<tr>
<td>6</td>
<td>[14C]GalNAc1-3Gal1-4GlcNAc...</td>
</tr>
<tr>
<td>7</td>
<td>GalNAc1-3Gal1-3GalNAc...</td>
</tr>
<tr>
<td>8</td>
<td>GalNAc1-3Gal1-4GlcNAc...</td>
</tr>
</tbody>
</table>

**Table I**

<table>
<thead>
<tr>
<th>Carbohydrate composition of human FVIII/vWF</th>
<th>Protein</th>
<th>Subunit</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residues</td>
<td>nmol/mg</td>
<td>mol/mol</td>
<td>%</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>120 ± 12</td>
<td>25 ± 3</td>
<td>3.7</td>
</tr>
<tr>
<td>Galactose</td>
<td>135 ± 13</td>
<td>28 ± 3</td>
<td>2.4</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>181 ± 18</td>
<td>38 ± 4</td>
<td>4.0</td>
</tr>
<tr>
<td>Mannose</td>
<td>175 ± 25</td>
<td>37 ± 5</td>
<td>3.2</td>
</tr>
<tr>
<td>Fucose</td>
<td>59 ± 6</td>
<td>12 ± 2</td>
<td>1.0</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>33 ± 4</td>
<td>7 ± 1</td>
<td>0.7</td>
</tr>
</tbody>
</table>

*a* Carbohydrate analysis was carried out as described under “Experimental Procedures.”

**Table II**

<table>
<thead>
<tr>
<th>Enzyme treatment</th>
<th>Minimum amount of glycoprotein required for hemagglutination inhibition</th>
<th>Blood group active structures expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>2nd</td>
<td>FVIII/vWF</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>800</td>
</tr>
<tr>
<td>GalNAcase</td>
<td>None</td>
<td>&gt;50,000</td>
</tr>
<tr>
<td>GalNAcase</td>
<td>GalNAc transferase</td>
<td>700</td>
</tr>
<tr>
<td>GalNAcase</td>
<td>GalNAc transferase</td>
<td>500</td>
</tr>
</tbody>
</table>

*a* Enzymatic modifications with limpet N-acetylgalactosaminidase (GalNAcase) or N-acetylgalactosaminyltransferase (GalNAc transferase) were performed as described under “Experimental Procedures.”

*h* Hemagglutination inhibition assays were performed using type A human erythrocytes and anti-A sera diluted to give twice the minimum concentration required for hemagglutination.
32 ± 4 nmol/mg of N-acetylgalactosamine. These values correspond to 95 and 97% of the respective amounts present before extraction (Table I). No amino sugars were detected in the organic phase. In a similar extraction of [14C]GalNAc-labeled FVIII/vWF, 93% of the recovered radioactivity was associated with the protein fraction. Thus, it seems unlikely that glycolipids could account for the blood group activity of FVIII/vWF. 

In order to confirm that blood group active oligosaccharide structures were covalently associated with the FVIII/vWF protein, [14C]GalNAc-labeled FVIII/vWF was analyzed by sodium dodecyl sulfate-gel electrophoresis (6) before and after reduction with β-mercaptoethanol. In Fig. 2, reduced unlabeled FVIII/vWF is on Gel A and reduced [14C]GalNAc-FVIII/vWF is on Gel B. Gel B contains two additional protein bands corresponding to the monomer and dimer of bovine serum albumin which was added to stabilize the N-acetylgalactosaminyltransferase. From the distribution of radioactivity shown for Gel B, it is apparent that [14C]GalNAc co-migrates primarily with the M, = 200,000 subunit of FVIII/vWF. After a similar analysis of the nonreduced samples, essentially all of the applied radioactivity was located at the extreme top of the gel along with the FVIII/vWF protein which does not significantly penetrate sodium dodecyl sulfate-5% acrylamide gels (6). These results demonstrate that [14C]GalNAc is incorporated into H blood group structures that are covalently attached to the FVIII/vWF polypeptide.

Digestion of [14C]GalNAc-Labeled FVIII/vWF with S. pneumoniae Endo-β-Galactosidase—The endo-β-galactosidase from S. pneumoniae (16) releases the A blood group trisaccharide, GalNAca1–3(Fucα1–2)Gal, when it is linked β1 → 4 to the penultimate sugar (type 2), but not when linked β1 → 3 (type 1) (Fig. 1). Elution profiles of [14C]GalNAc-labeled FVIII/vWF on Sephadex G-25 before and after digestion with the endosidase are shown in Fig. 3. It is apparent that a low molecular weight product containing [14C]GalNAc is released from FVIII/vWF. The results of endosidase treatment of [14C]GalNAc-labeled derivatives of porcine submaxillary mucin and asialo-α1-acid glycoprotein are also shown (Fig. 3). In keeping with the specificity of the endosidase, C-labeled oligosaccharides were not released from porcine submaxillary mucin which carries the sequence [14C]GalNAcα1–3(Fucα1–2)Galβ1 → 3GalNAc-R, but labeled oligosaccharides were quantitatively released from the derivative of asialo-α1-acid glycoprotein containing the terminal sequence [14C]GalNAcα1–3(Fucα1–2)Galβ1 → 4GlcnAc-R.

For further characterization, products released from large scale endosidase digests of [14C]GalNAc-FVIII/vWF and [14C]GalNAc-α1-acid glycoprotein were isolated as described under “Experimental Procedures.” Products from both proteins eluted from a gel filtration column of Bio-Gel P-4 at positions expected for a neutral trisaccharide. These were compared by paper chromatography with oligosaccharide standards including authentic A blood group active trisaccharide [14C]GalNAc(Fucα1–2)Gal prepared by endosidase treatment of [14C]GalNAc-labeled furcosylactosamine (Fig. 1). As shown in Fig. 4, the endosidase-released products from both [14C]GalNAc-labeled glycopeptides co-migrate with the A blood group active trisaccharide [14C]GalNAcα1–3(Fucα1–2)Gal.

These results indicate that the [14C]GalNAc-labeled H blood group sites of FVIII/vWF are linked β1 → 4 to the penultimate sugar. Moreover, the endosidase was also able to release all the [14C]GalNAc incorporated into the N-acetylgalactosaminidase-treated derivative of FVIII/vWF. Thus, since this labeled derivative reflects both the A and H blood group structures present on native FVIII/vWF, it appears that the A blood group structures of native FVIII/vWF are also linked β1 → 4 to the penultimate sugar.

Alkaline Borohydride Treatment of [14C]GalNAc-Labeled FVIII/vWF—In order to determine if the blood group active structures of FVIII/vWF are components of threonine/serine or asparagine-linked oligosaccharides, [14C]GalNAc-labeled FVIII/vWF was subjected to alkaline borohydride reduction and analyzed by gel filtration on Sephadex G-50. For comparison, [14C]GalNAc-labeled asialo-porcine submaxillary mucin, which contains [14C]GalNAcα1–3(Fucα1–2)Galβ1 → 3GalNAc linked to threonine/serine as the only labeled oligo-

![Fig. 2. Sodium dodecyl sulfate-5% polyacrylamide gel electrophoresis of [14C]GalNAc-FVIII/vWF. Gels containing 5% acrylamide and stained with Coomassie blue are shown; Gel A contains 8 μg of reduced FVIII/vWF and Gel B, 8 μg of reduced [14C]GalNAc-labeled FVIII/vWF. Bovine serum albumin observed on Gel B was added to stabilize the transferase during incorporation of [14C]GalNAc. The graph shows the distribution of [14C]GalNAc corresponding to Gel B. Radioactivity was determined after slicing the gel into 3-mm sections and incubating each with 1.0 ml Protosol for 12 h at 55°C. The amount of applied radioactivity recovered was 92%.](http://www.jbc.org/)

![Fig. 3. Gel elution profiles of [14C]GalNAc-labeled glycoproteins before (−) and after (+) digestion with S. pneumoniae endo-β-galactosidase. [14C]GalNAc-FVIII/vWF, [14C]GalNAc-porcine submaxillary mucin (PSM); and [14C]GalNAc-α1-acid glycoprotein were prepared as described under “Experimental Procedures.” Aliquots of each were incubated for 16 h at 37°C either with (+) or without (−) approximately 5 milliunits of S. pneumoniae endo-β-galactosidase in a total volume of 80 μl of 0.125 M sodium cacodylate, pH 6.0, containing 125 μg bovine serum albumin. Each reaction mixture was applied to a column (1.5 x 23 cm) of Sephadex G-25 (superfine) preconditioned with a 100-mg sample of bovine serum albumin and equilibrated with 0.15 M sodium chloride. One-milliliter fractions were collected directly into scintillation vials and counted.](http://www.jbc.org/)
Carbohydrate on Human Factor VIII/von Willebrand Protein

stable under these conditions. After incubation for 0.5 or 6 h the low molecular weight product eluted at the position of a tetrasaccharide on a calibrated column of Sephadex G-25 (superfine) equilibrated in 0.15 M sodium chloride (data not shown). The incubation conditions were sufficient for complete reduction of the β-eliminated product since no evidence of base-catalyzed degradation products was detected. In contrast, alkaline borohydride treatment of [14C]GalNAc-labeled FVIII/vWF produced low molecular weight products within 0.5 h that were further degraded to smaller products upon continued incubation. The profile of the [14C]GalNAc-labeled products from the limit digest of the FVIII/vWF at 20 h was virtually identical to that at 6 h. Of interest, these products eluted earlier than an asparagine-linked glycopeptide (designated McM) of approximately M, = 2000 (27) (Fig. 5) and have an approximate molecular weight of 3100 ± 400. Thus, when subjected to alkaline borohydride treatment, the degradation pattern of the [14C]GalNAc-labeled II blood groups

glycosaccharide, was examined in parallel experiments. As shown in Fig. 5, both [14C]GalNAc-labeled proteins elute in the void volume of the column prior to treatment. After 6 h of alkaline borohydride treatment, the [14C]GalNAc label from either protein elutes with low molecular weight product(s) in the included volume of the column. Although these results would be consistent with the release of O-linked oligosaccharides from threonine or serine, the changing pattern of degradation of [14C]GalNAc-labeled FVIII/vWF with time does not support this interpretation.

The pattern of degradation characteristic of reductive β-elimination of O-linked oligosaccharides is illustrated by the results with [14C]GalNAc-labeled porcine submaxillary mucin (Fig. 5). With increasing periods of incubation, a low molecular weight product of discrete size accumulates as the released oligosaccharide is immediately reduced and is subsequently

FIG. 4. Paper chromatograms of the products released from [14C]GalNAc-labeled glycoproteins upon digestion with S. pneumoniae endo-β-galactosidase. Products released from endo-β-galactosidase digestion of [14C]GalNAc-labeled derivatives were prepared for chromatography as described under "Experimental Procedures." Samples were applied to a sheet of Whatman No. 3MM (45 x 56 cm) and developed by descending chromatography for 87 h with butanol:ethanol:water (10:1:2). Arrows in Panel A indicate the positions of oligosaccharide standards located with the silver nitrate stain for reducing sugars (26) including (2) lacto-N-tetraose, (2) fucosyllactose, (3) lactose, (4) Galβ1 → 3GalNAc, (5) Galβ1 → 4GalNAc, and (6) GalNAc. Lanes containing radiolabeled products were cut into 0.25-cm strips and counted in 5-ml toluene containing 4 g of 2,5-diphenyloxazole/liter. Profiles shown are for [14C]GalNAc-fucosyllactose (Panel A) and the products released from endo-β-galactosidase digestions of [14C]GalNAc-fucosyllactose (Panel B), [14C]GalNAc-thracid glycoprotein (Panel C), and [14C]GalNAc-FVIII/vWF (Panel D).

FIG. 5. Gel filtration elution profiles of [14C]GalNAc-FVIII/vWF and [14C]GalNAc-porcine submaxillary mucin (PSM) after treatment with alkaline borohydride. [14C]GalNAc-labeled derivatives were prepared as described under "Experimental Procedures" and aliquots containing about 3000 cpmp were incubated at 40 °C in 200 μl of 0.1 N NaOH containing 0.8 M NaBH₄. At various times, reaction mixtures were neutralized by adding galacial acetic acid and stored at 4 °C. Gel filtration was performed on a column (1.5 x 28 cm) of Sephadex G-50 (fine) pretreated with 50 mg of bovine serum albumin and equilibrated in 0.15 M sodium chloride. Fractions (1.2 ml) were counted to locate radiolabeled products. Elution profiles after increasing periods of incubation with alkaline borohydride are shown on the left for [14C]GalNAc-porcine submaxillary mucin, and on the right for [14C]GalNAc-FVIII/vWF. The times of incubation for each sample are indicated in the corresponding panel. The arrow in the bottom right panel designates the elution position of the McM glycopeptide which has a molecular weight of about 2000 (27). The column void and included volumes are designated V₀ and Vₑ, respectively.
of FVIII/vWF is not typical of oligosaccharides O-linked to threonine or serine residues.

Since alkaline borohydride treatment has been shown to degrade polypeptide structures (28), it was of interest to determine whether asparagine-linked oligosaccharides of glycoproteins would also appear as products of low molecular weight. Accordingly the asparagine-linked oligosaccharides of asialo-α1-acid glycoprotein were labeled by incorporation of [14C]GalNAc with the β-galactoside α2 → 6 sialyltransferase as described previously (23). Aliquots containing 0.1 mg (20,000 cpm) were chromatographed on a column (1.2 × 24 cm) of Sephadex G-50 (fine) in 0.15 M NaCl either before (●) or after (○) treatment (18 h) with alkaline sodium borohydride as described in Fig. 6. Panel A, asialo-al-acid glycoprotein was labeled with [14C]GalNAc-FVIII/vWF was labeled with [3H]-NeuAc by glycosylation with the β-galactoside α2 → 6 sialyltransferase as previously described (23). Aliquots containing 0.1 mg (20,000 cpm) were chromatographed on a column (1.2 × 24 cm) of Sephadex G-50 (fine) in 0.15 M NaCl either before (●) or after (○) treatment (18 h) with alkaline sodium borohydride as described in Fig. 6. Panel B, [14C]GalNAc-FVIII/vWF was labeled with [3H]-NeuAc by incubation with CPM-[3H]NeuAc and β-galactoside α2 → 6 sialyltransferase as described under “Experimental Procedures.” Approximately 0.2 mg containing 42,500 cpm [3H]NeuAc and 5000 cpm of [14C]GalNAc was subjected to alkaline borohydride treatment for 20 h and gel filtered on a column (1.5 × 28 cm) of Sephadex G-50 (fine) as described in Fig. 5. Fractions (1.2 ml) were measured for radioactivity using double label counting procedures. Approximately 88% of both the applied [3H] and [14C] counts were recovered.

DISCUSSION

In this report, A, B, and H blood group structures are identified on oligosaccharides of human FVIII/vWF isolated from pooled human plasma. To our knowledge, A, B, and H oligosaccharide structures have not been reported on other glycoproteins isolated from plasma. Indeed, while as little as 0.8 μg of FVIII/vWF inhibited hemagglutination by anti-A sera, no inhibition was observed with 1000 μg of α1-acid glycoprotein, transferrin, fetuin, or IgG (Table II). Our value of 15% carbohydrate is intermediate between the 5.8% reported for the human protein by others (30) and the 19.8% obtained for bovine FVIII/vWF (31). Moreover, our results indicate that monosaccharides typical of both asparagine- and threonine- or serine-linked oligosaccharides comprise the carbohydrate on human FVIII/vWF (Table I). The blood group structures isolated from FVIII/vWF represent only a small portion of the total carbohydrate. The estimated values of 1.4 mol of H and 0.7 mol of A blood group structures/mol of M, = 200,000 subunit requires only 18, 8, and 10% of the fucose, galactose, and N-acetylgalactosamine contents of FVIII/vWF, respec-
tively. It is of particular interest that the blood group oligosaccharides, even though present as a small fraction of the total carbohydrate, could be detected and quantitated by the use of specific enzymes capable of modifying such structures.

Previous structural studies have shown that blood group structures (Fig. 1) occur at the nonreducing termini of glycoprotein and glycolipid oligosaccharides and are linked $\beta_1 \rightarrow 3$ (type 1) or $\beta_1 \rightarrow 4$ (type 2) to the penultimate sugar, either N-acetylgalactosamine or N-acetylgalactosaminic (32). In this report, the A and H oligosaccharides of FVIII/vWF were radiolabeled by first converting the A structures to $H$ (Fucal $\rightarrow 2$Gal) with N-acetylgalactosaminidase, and then incorporating $^{[14]}$CGalNAc into the nascent and newly formed H structures with N-acetylgalactosaminyltransferase to form the A blood group structure $^{[14]}$CGalNAc (Fucal $\rightarrow 2$Gal). All of the $^{[14]}$C label could be released by the S. pneumoniae- $\beta$-galactosidase as the trisaccharide $[''Cl-GalNAc(Fucal \rightarrow 2)Gal$. This enzyme releases the A blood group trisaccharide only when linked $\beta_1 \rightarrow 4$ to the penultimate sugar and not when linked $\beta_1 \rightarrow 3$ (16). Thus, the A and H structures of FVIII/vWF are of type 2 (Fig. 1).

The A and H structures of type 2 have been shown to be components of oligosaccharides of ovarian cyst mucin which are O-linked to threonine or serine (32). Although blood group structures attached to N-linked oligosaccharides have not been reported previously (24), the possibility that they could exist is amply demonstrated by our ability to form in vitro both A and H structures on the asparagine-linked oligosaccharides of asialo-$\alpha_1$-acid glycoprotein (Fig. 1) when sequentially glycosylated with the $\beta$-galactoside $\alpha_1 \rightarrow 2$ fucosyltransferase and the N-acetylgalactosaminyltransferase (23). Moreover, the resulting H and A structures are of type 2 since the terminal galactose residues of asialo-$\alpha_1$-acid glycoprotein are linked $\beta_1 \rightarrow 4$ to N-acetylgalactosamine. The release of low molecular weight oligosaccharides under conditions of $\beta$-elimination is commonly interpreted as characteristic of O-linked oligosaccharides. However, while O-linked oligosaccharides are released as a discrete molecular unit, the products of alkaline borohydride treatment of FVIII/vWF exhibit a time-dependent decrease in size. As also shown in this report with $\alpha_1$-acid glycoprotein, the conditions used for $\beta$-elimination can result in the production of low molecular weight N-linked oligosaccharides, presumably by degrading the polypeptide chain. Thus, these experiments suggest that the blood group oligosaccharides of FVIII/vWF may be in an alkaline stable linkage to the polypeptide.

The implications of our results with regard to the in vivo function of this protein after infusion of FVIII/vWF concentrates is not clear. In preliminary experiments, no inhibition of either procoagulant activity or ristocetin-induced, platelet-aggregating activity was observed by preincubation of the purified protein with A or B anti-sera. Moreover, no precipitin reaction was observed following immunodiffusion analyses. In the latter case, the low concentration of blood group structures (1 to 2 mol/mol) may not provide sufficient valency to support a precipitin reaction. The cause of spontaneous formation of antibodies to human FVIII/vWF in some hemophiliacs after repeated transfusions of FVIII/vWF concentrates from pooled plasma is unknown. It may prove fruitful to determine if a relationship exists between the level of blood group active structures in pooled preparations of FVIII/vWF concentrates, the blood types of hemophiliacs who receive such concentrates, and the tendency for such patients to form antibodies to FVIII/vWF.

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