The Structure of a Phytohemagglutinin Receptor Site from Human Erythrocytes*

Rosalind Kornfeld and Stuart Kornfeld

From the Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

(Received for publication, January 8, 1970)

SUMMARY

A highly purified glycopeptide with potent phytohemagglutinin (PHA) receptor site activity has been isolated from human erythrocyte membranes. The glycopeptide was released from the membranes by trypsin, treated with alkaline borohydride, and purified by repeated gel filtration, further proteolytic digestion with Pronase, and diethylaminoethyl cellulose chromatography. It has a molecular weight of approximately 2000 and the following composition (in residues): sialic acid, 1; galactose, 3; mannose, 3; N-acetylglucosamine, 3; aspartic acid, 1.5; serine, 1; and threonine, 1. The sequence of the carbohydrate residues in the oligosaccharide chain was determined by sequential cleavage of the sugars from the nonreducing end with specific glycosidases. The glycopeptide has a single branched oligosaccharide chain containing two nonreducing termini, one with the composition galactose-N-acetylglucosamine and the other with sialic acid-galactose-N-acetylglucosamine. Each branch is connected to the inner core which contains 2 mannose and 1 N-acetylglucosamine residues. Since the oligosaccharide chain was not released from the tryptic fragment during the alkaline borohydride treatment, it is probably linked to the peptide backbone by an N-acetylgalactosaminylasparagine linkage.

Removal of the sialic acid residue does not affect PHA-inhibitory activity. When the galactose residues are removed, 90% of the PHA-inhibitory activity is lost. Model compounds having the terminal sequences galactose, N-acetylglucosamine have very poor PHA-inhibitory activity unless they also have mannose residues in their interior, showing that the inner core sugars can influence PHA-inhibitory activity. The peptide backbone of the glycopeptide also affects the PHA-inhibitory activity of the oligosaccharide chain since higher molecular weight glycopeptides extracted from erythrocyte membranes with chloroform-methanol lose 90% of their PHA-inhibitory activity following proteolytic digestion with trypsin.

* This research was supported in part by Grants HE-0022-22 and RO1 CA-08759-01 from the United States Public Health Service and Grant PRA-31 from the American Cancer Society.

1 The abbreviation used is: PHA, phytohemagglutinin.
Preparation of Erythrocyte Glycopeptide

**Step 1: Trypsin Treatment of Erythrocytes**—The erythrocytes from 3 liters of outdated bank blood were washed three times with 3 volumes of 0.9% NaCl-0.01 M NaHCO₃ to remove the plasma and buffy coat, and then the erythrocytes were treated with trypsin as described by Wissler et al. (5). One volume of packed red cells was added to 1 volume of 0.9% NaCl-0.05 M phosphate buffer, pH 7.5, containing 0.25 mg per ml of trypsin 1/250 (Difco). The cell suspension was incubated with shaking at 37° for 1 hour during which time essentially no cell lysis occurred. The cells were then removed by centrifugation and to the chilled supernatant fluid one-eighth volume of cold 50% trichloroacetic acid was added. The resulting precipitate was removed by centrifugation at 15,000 X g for 10 min and the supernatant fluid, containing all the trypsin-released glycopeptides, was neutralized with NaOH, dialyzed overnight at 4°, and then lyophilized.

**Step 2: Alkaline Borohydride Treatment**—The trypsin-released glycopeptides from 1200 ml of red cells were incubated (total volume, 15 ml) with 0.1 M sodium borohydride in 0.2 N NaOH for 36 hours at room temperature under an N₂ atmosphere. The solution was then neutralized with 2 N HCl and concentrated to 3 ml.

**Step 3: Gel Filtration**—The concentrated material from the alkaline borohydride treatment was applied to a Sephadex G-25-80 column (1.2 x 33 cm). The column was eluted with water and 1.5-ml fractions were collected. The glycopeptide material which appeared in the exclusion volume of the column contained all the PHA inhibitory activity but only one-third of the sialic acid. This active material from three identical preparations was concentrated and applied to a Sephadex G-75-40 column (1.5 x 80 cm) (Fig. 1). Elution was carried out with water and 3.0-ml fractions were collected. The active glycopeptides were combined into Fractions A and B as shown in Fig. 1.

**Step 4: Pronase Digestion**—The A fractions and the B fractions from four identical Sephadex G-75 columns were each combined (representing material derived from 12 preparations totaling 14,200 ml of packed erythrocytes) and concentrated to a volume of 10 ml. The two fractions (A and B) were each incubated with 15 mg of Pronase in 0.05 M Tris buffer, pH 7.8, containing 0.002 M CaCl₂ in a final volume of 15 ml. Incubation was carried out for 66 hours at 37° under a toluene atmosphere. The reaction mixtures were then heated in a boiling water bath for 4 min and cooled. This treatment caused less than 5% loss of PHA inhibitory activity of Fraction B and approximately a 15% loss of activity of Fraction A.

**Step 5: Repeat Gel Filtration**—The A and B glycopeptide reaction mixtures were reduced to a volume of 3 ml and applied
formed as before. Z.U., inhibitory unit.

In Fig. 3 was rechromatographed on DEAE-cellulose using as the eluting buffer the lowest concentration of phosphate buffer, pH 6.8, calculated to displace the glycopeptide. Analyses for sialic acid (●) and PHA inhibitory activity (O—O) were performed as before. I.U., inhibitory unit.

to a Sephadex G-50 column (1.5 x 80 cm) (Fig. 2). Elution was carried out with water and 2.5-m1 fractions were collected. The A glycopeptide material contained one main peak of PHA inhibitory activity while the B glycopeptide material resolved into three active peaks (I, II, and III).

Step 6: DEAE-cellulose Chromatography—The B-II fraction from the Sephadex G-50 column was concentrated and loaded onto a column (0.9 x 11.5 cm) of DEAE-cellulose that had been equilibrated with 3 mM sodium phosphate buffer (pH 6.8). The column was washed with 30 ml of the equilibrating buffer and eluted with a linear gradient (160 ml) from 3 to 100 mM phosphate buffer, pH 6.8 (Fig. 3). The column was then washed with 200 m1 phosphate buffer, pH 6.8. Fractions of 3.5 ml were collected. The active fractions from each of the four peaks (II-D-1, II-D-2, II-D-3, and II-D-4) were pooled.

Step 7: Repeat DEAE-cellulose Chromatography—The II-D-3 fraction was concentrated, dialyzed against water, and then loaded onto another DEAE-cellulose column that had been equilibrated with 3 mM phosphate buffer, pH 6.8. The column was washed with 45 ml of the starting buffer and then successively eluted with 65 ml of 5 mM phosphate buffer, 105 ml of 7 mM phosphate buffer, and finally 35 ml of 50 mM phosphate buffer (all pH 6.8) (Fig. 4).

Chemical Determinations

Sialic acid was measured by the method of Warren (6) following hydrolysis in 0.05 N H2SO4 for 1 hour at 80° (or 1 N HCl for 1 min at 100° (7)). Total hexose was measured by the phenolsulfuric acid method scaled down to one-fifth volume (8). Individual neutral sugars were determined following hydrolysis in 2 N H2SO4 for 4 hours at 100° and paper chromatography in 1-butanol-ethanol-water (10:1:2) (Solvent I). Recovery was corrected for by including 14C tracer sugars in the hydrolysis. Galactose and mannose were measured by the Park-Johnson ferricyanide method (9) and fucose by the cysteine-H2SO4 method (10). Both tests were scaled down to one-fifth volume. Hexosamine was determined with a Spinco automatic amino acid analyzer following hydrolysis in 4 N HCl for 4 hours at 100° in a vacuum and lyophilization to remove HCl. N-Acetylglucosamine released by β-N-acetylgalactosaminidase was measured by the method of Reissig, Strominger, and Leloir (11). Quantitative estimates of amino acid composition were obtained with the amino acid analyzer following hydrolysis of the glycopeptide in constant boiling HCl for 16 hours at 105° in an evacuated sealed tube.

Enzymes

Neuraminidase (EC 3.2.1.18) from Vibrio cholerae and Pronase were purchased from Calbiochem. Trypsin 1/250 was purchased from Difco. β-Galactosidase (EC 3.2.1.23), β-N-acetylgalactosaminidase (EC 3.2.1.30), and α-mannosidase (EC 3.2.1.24) were prepared from jack bean meal by a slight modification of the method of Li (12). The β-galactosidase preparation was free of β-N-acetylgalactosaminidase and α-mannosidase activities while the β-N-acetylgalactosaminidase preparation was free of β-galactosidase and α-mannosidase. Assays for the glycosidases were carried out in 0.05 M citrate buffer, pH 4.6; the substrates were, for β-galactosidase, 10 mM α-nitrophenyl β-galactoside; for β-N-acetylglucosaminidase, 1 mM p-nitrophenyl N-acetyl-β-glucosaminide; for α-mannosidase, 1 mM p-nitrophenyl α-mannopyranoside. β-Galactosidase assays were carried out at 37° and the others at 25°. A unit of activity in each case was defined as the amount of enzyme which could liberate 1.0 μmole of α- or p-nitrophenol in 60 min.

Action of Glycosidases on Glycopeptides

Because of the limited amount of purified glycopeptide that was available, the following scheme was used during the sequen- tial enzyme treatment of the purified glycopeptide. To 0.1 to 0.2 μmole of glycopeptide in 0.05 M citrate buffer, pH 4.6, was added either β-galactosidase (3 units), β-N-acetylgalactosaminidase (8 units), or α-mannosidase (15 units). The reaction mixture (0.2 ml) was incubated at 37° under a toluene atmosphere for a specified number of hours and then heated in a boiling water bath for 2 min. Water and tracer 14C sugars (galactose and mannose) were added to the heated reaction mixture to bring the volume to 0.5 ml and the precipitated enzyme protein was removed by centrifugation. The supernatant fluid was then applied to a previously calibrated Sephadex G-25 fine column (0.9 x 9.0 cm) (Ve = 2.5 ml) to separate the residual glycopeptide (in the exclusion volume) from the released sugar residues (which were retarded). The residual glycopeptide, which was recovered quantitatively, was evaporated to dryness, redissolved in 0.05 M citrate buffer, pH 4.6, and reincubated with the same glycosidase. This process was repeated until no more sugar was released (two incubations were usually adequate) and then the residual glycopeptide was incubated with another glycosidase. When release of galactose or mannose was being measured the released sugar residues plus the tracer 14C sugars were deionized with an Amberlite MB-3 mixed bed resin, evaporated to dryness, and then spotted on Whatman No. 1 paper and chromatographed in Solvent I. The released sugars were quantitatively determined as described above. When release of N-acetylgalactosamine was being measured an aliquot of the retarded fraction was analyzed directly. In this manner serial incubations could be performed on a single glycopeptide sample. In some experiments N-acetylgalactosamine release was followed...
by testing aliquots removed directly from the incubating reaction mixture.

**Chloroform-Methanol Extraction of Erythrocyte Ghosts**

Washed red cells were lysed with 9 volumes of water and sedimented at 12,000 × g for 12 min. The packed ghosts were then washed four times with 0.01 m Tris-0.0001 m EDTA, pH 7.4, to remove residual hemoglobin. For every milliliter of packed "ghosts,” 9 ml of chloroform-methanol (2:1) were added with vigorous shaking of the flask. The mixture was stirred for 30 min and the aqueous layer was carefully removed and concentrated in a rotary evaporator.

**Glycopeptides and Oligosaccharides**—The transferrin glycopeptide containing a single asparagine residue was a gift of Dr. Graham Jamieson (13). Lacto-N-tetraose, lacto-N-neotetraose, and lactotetraose were kindly provided by Drs. Kobata and Ginsburg (National Institutes of Health). Fetuin was purchased from Grand Island Biological Company (Grand Island, New York) and the fetuin glycopeptides isolated by Sephadex gel filtration following Pronase digestion as described by Spiro (14).

**RESULTS**

**Isolation of Glycopeptides with Phytohemagglutinin Inhibitory Activity**—Trypsin treatment of intact human erythrocytes released from the cell membrane a number of glycopeptides which were capable of binding to erythroagglutinating PHA and inhibiting the agglutination of red cells by that molecule. When the trypsin-released glycopeptides were treated with alkaline borohydride, approximately two-thirds of the sialic acid-containing oligosaccharides were released from the peptide backbone (presumably those chains linked an-glycosidically to the hydroxyl groups of serine and threonine (15)). The residual glycopeptide material, which eluted in the exclusion volume of the Sephadex G-25 column, retained almost full inhibitory activity. This material was separated into two main components (A and B) upon subsequent gel filtration on Sephadex G-75 (Fig. 1). When the active glycopeptides in Peaks A and B were subjected to proteolytic digestion with Pronase followed by Sephadex G-50 gel filtration, the patterns seen in Fig. 2 were obtained. The A glycopeptides resolved into several sialic acid-containing peaks, only one of which had PHA inhibitory activity. However, chromatography of this active material on DEAE-cellulose revealed that it contained at least five active components. Since the amount of glycopeptide material in these fractions was quite limited (compare Peaks A and B of Fig. 1), further purification steps were not undertaken.

The Pronase-treated B glycopeptides separated into three active fractions (I, II, and III) on Sephadex G-50 (Fig. 2). When each of these fractions was subjected to DEAE-cellulose chromatography, each was resolved into a number of active components. Since the most highly purified glycopeptide with PHA inhibitory activity was derived from the Peak II material, the subsequent description of the purification scheme will be limited to this material.

Chromatography of the B-II material on DEAE-cellulose resulted in the separation of a number of sialic acid-containing glycopeptides as shown in Fig. 3. Only two of these glycopeptides had significant PHA inhibitory activity while others, such as the material eluted with the 0.2 m phosphate buffer, were almost completely inactive. The active glycopeptides which eluted from the column when the conductivity of the phosphate buffer was 0.55 millimho were combined (designated II-D-3), dialyzed, and rechromatographed on DEAE-cellulose (Fig. 4). Batch elution with 5 m phosphate buffer, pH 6.8 (conductivity of 0.55 millimho), resulted in the separation of this glycopeptide from another active, but more negatively charged glycopeptide. The glycopeptide (II-D-3) which eluted from the DEAE-cellulose column with the 5 m phosphate buffer contained a total of 27,500 PHA inhibitory units, representing a recovery of about 3.7% of the total inhibitory activity in the starting material. The data in Table I summarize the recovery of inhibitory activity during the various stages of purification of II-D-3.

**Composition of Glycopeptide II-D-3**—The composition of the most highly purified glycopeptide is shown in Table II. The molecular weight of the glycopeptide would be approximately 2000 based on the chemical composition and assuming a single oligosaccharide chain. When II-D-3 was subjected to gel filtration on Sephadex G-50, it was more retarded than a purified transferrin glycopeptide with a molecular weight of approximately 2500 (13). This finding is consistent with the proposal that II-D-3 has a single oligosaccharide chain. The fact that the oligosaccharide chain of the glycopeptide had not been released during the alkaline borohydride treatment of the original trypsin fragment suggests that it is linked to the peptide through a glycosylamine-type linkage between asparagine and N-acetylglucosamine. The glycopeptide (II-D-3) which eluted from the DEAE-cellulose column with the 5 m phosphate buffer contained a total of 27,500 PHA inhibitory units, representing a recovery of about 3.7% of the total inhibitory activity in the starting material. The data in Table I summarize the recovery of inhibitory activity during the various stages of purification of II-D-3.

**Action of Glycosidases on II-D-3**—When the II-D-3 glycopeptide was treated successively with β-galactosidase and β-N-acetylgalactosaminidase, 0.9 residue of galactose and 0.9 residue of N-acetylgalactosamine were released per oligosaccharide chain (Table III). Treatment of the glycopeptide with β-N-acetylgalactosaminidase alone released only 0.12 residue of N-acetylgalactosamine. These results indicated that the oligosaccharide chain of II-D-3 contains a nonreducing end with the sequence galactose → N-acetylgalactosamine → X. When the terminal sialic acid residue of II-D-3 was removed by mild acid hydrolysis (desialized II-D-3 in Table III) and then the sequential enzyme treatment was carried out, 1.7 residues of galactose and 2.0 residues of N-acetylgalactosamine were released. Since galactose residues and 1 N-acetylgalactosamine residue are contained in the nonreducing end just described, the remaining galactose and N-acetylgalactosamine residues must be in a second chain terminating in sialic acid which has the sequence sialic acid → galactose → N-acetylgalactosamine → Y. While the galactose residues were released relatively rapidly (9 hours incubation time), the N-acetylgalactosamine residues were released at a much slower rate (Fig 5). However, even after prolonged digestion, no more than 0.9 and 2.0 residues of N-acetylgalactosamine were released from II-D-3 and desialized II-D-3, respectively.

The release of galactose from II-D-3 and desialized II-D-3 by β-galactosidase was accompanied by a loss of PHA inhibitory activity. The amount of inhibitory activity lost was proportional to the amount of galactose liberated at intermediate time intervals and after completion (9 hours) II-D-3 retained 32% of its original activity and desialized II-D-3 only 13% of its original activity. These results showed the importance of the galactose residues in the binding of the glycopeptide to PHA. Removal of the sialic acid residue did not result in a loss of PHA inhibitory activity.

The glycopeptide did not release any mannose when treated...
initially with \( \alpha \)-mannosidase. When II-D-3 was treated successively with \( \beta \)-galactosidase, \( \beta \)-acetylglucosaminidase, and then with \( \alpha \)-mannosidase, 0.32 residue of mannose was released from desialized II-D-3 during the mild acid hydrolysis required to remove the sialic acid. Based on the total galactose in desialized II-D-3 liberation of 1.7 residues corresponds to 89% released.

**Fig. 5.** Enzymatic removal of \( \beta \)-N-acetylglucosaminidase (\( \text{GlcNAc} \)) residues from glycopeptide II-D-3. Glycopeptide II-D-3 and a sample of desialized II-D-3 were exhaustively treated with \( \beta \)-galactosidase to remove all of the terminal galactose residues. Then the purified \( \beta \)-N-acetylglucosaminidase was added to give 42 units per ml and the mixtures incubated in 0.05 M citrate buffer, pH 4.6, at 37° under a toluene atmosphere. In Experiment 1 (○ and □), each glycopeptide was present at a concentration of 0.5 \( \mu \)mole per ml and in Experiment 2 (● and ◼), at a concentration of 1.0 \( \mu \)mole per ml. The amount of N-acetylglucosamine released at each time shown was measured on an aliquot of the reaction mixtures and has been expressed as the number of residues liberated per molecule of glycopeptide so that both experiments are comparable. ○ and □, amount released from II-D-3; ● and ◼, amount released from desialized II-D-3.

---

**Table I**

Summary of purification of glycopeptide II-D-3

| Fraction | Total inhibitory units | Total sialic acid | Yield % |
|----------|------------------------|-------------------|---------|
| 1. Trypsin-released glycopeptides... | 750,000 | ~1,600 | 100 |
| 2. Alkaline borohydride treatment followed by Sephadex G-25 | 580,000 | 475 | 77 |
| 3. Sephadex G-75 | 117,250 | 35 | 15.7 |
| 4. Pronase digestion of B followed by Sephadex G-50 | 71,300 | 43 | 6.0 |
| 5. DEAE-cellulose column of II | 124,500 | 85 | 16.6 |
| 6. Second DEAE-cellulose column of II-D-3 | 27,500 | 6.4 | 3.7 |

**Table II**

Composition of glycopeptide II-D-3

Carbohydrate and amino acid analyses were carried out as described under "Experimental Procedure."

| Molar ratio to mannose | µmol/ml | Actual | Nearest half integer |
|------------------------|---------|--------|---------------------|
| Sialic acid            | 1.0     | 1.1    | 1                   |
| Hexose                 | 3.6     | 3.9    | 4                   |
| Galactose              | 1.9     | 2.1    | 2                   |
| Mannose                | 1.85    | 2.0    | 2                   |
| Fucose                 | 0.055   | 0.06   | 0                   |
| N-Acetylglucosamine    | 3.06    | 3.3    | 3                   |
| N-Acetylgalactosamine  | <0.1    | 0.0    | 0                   |
| Aspartic acid          | 1.45    | 1.57   | 1.5                 |
| Serine                 | 1.10    | 1.2    | 1                   |
| Threonine              | 0.86    | 0.83   | 1                   |
| Glutamic acid          | 0.46    | 0.50   | 0.5                 |
| Alanine                | 0.26    | 0.28   | 0.3                 |
| Valine                 | 0.25    | 0.27   | 0.3                 |

---

**Table III**

Effect of glycosidases on glycopeptide II-D-3

II-D-3 (0.2 \( \mu \)mole) and desialized II-D-3 (0.2 \( \mu \)mole) were digested exhaustively with glycosidases in the following sequence: Experiment 1, \( \beta \)-galactosidase, \( \beta \)-N-acetylglucosaminidase, and \( \alpha \)-mannosidase; Experiment 2, \( \beta \)-N-acetylglucosaminidase alone; Experiment 3, \( \alpha \)-mannosidase alone. The details of the enzyme digestions and analysis of released sugars are given under "Experimental Procedure." II-D-3 was desialized by mild acid hydrolysis at pH 2 for 1 hour at 80°, and filtered over Sephadex G-25 to remove the released sialic acid.

| Enzyme                  | II-D-3 | Desialized II-D-3 |
|-------------------------|--------|-------------------|
| µmol released/µmol total |        | %                |
| µmol released/µmol total |        | %                |

* This percentage value (based on total composition of II-D-3) is low because it was shown that a small amount of galactose was removed from desialized II D 3 during the mild acid hydrolysis required to remove the sialic acid. Based on the total galactose in desialized II-D-3 liberation of 1.7 residues corresponds to 89% released.
Effect of periodate oxidation on monosaccharides of glycopeptide II-D-3

An aliquot of glycopeptide II-D-3 containing 0.1 μmole of sialic acid was incubated at 4°C in the dark with 4 μmoles of sodium metaperiodate in 0.05 M acetate buffer, pH 4.6, in a volume of 0.15 ml. A control tube contained no periodate. After 18 or 66 hours, 8 μmoles of ethylene glycol were added to react with the excess periodate (25 min at 25°C). The control tube was added 4 μmoles of periodate that had been previously incubated 25 min at 25°C with 8 μmoles of ethylene glycol. Each sample was then subjected to acid hydrolysis with tracer 14C-galactose, mannose, and, in Experiment 1, glucosamine and the sugars separated and quantitatively determined as described under "Experimental Procedure." The amount of each sugar in the oxidized samples is expressed as a percentage of the amount in the controls (0 hours of oxidation).

| Length of oxidation (hrs) | Galactose | Mannose | N-Acetylglucosamine |
|--------------------------|-----------|---------|---------------------|
| Experiment 1             | 100       | 100     | 100                 |
| 18                       | 2         | 25      | 92                  |
| Experiment 2             | 100       | 100     | ND*                 |
| 66                       | 23        | 38      | ND                  |

* ND, not determined.

The nonreducing termini have been degraded (Table III, desialized II-D-3).

Periodate Oxidation of II-D-3—Periodate oxidation of the glycopeptide resulted in marked destruction of the galactose and mannose residues but no significant destruction of N-acetylglucosamine (Table IV).

Binding of Glycopeptide II-D-3 to Phytohemagglutinin—The glycopeptide II-D-3 binds specifically to phytohemagglutinin as shown in Fig. 6. In this experiment phytohemagglutinin, but not albumin, was able to displace the glycopeptide from its usual elution position on Sephadex G-75 to the exclusion volume of the column. The data also indicate that at least 71% of the glycopeptide material is capable of binding to phytohemagglutinin, thus providing an estimate of the purity of the glycopeptide preparation. Previous incubation of the glycopeptide with PHA for a longer time (3 or 5 hours) did not increase the amount of II-D-3 bound. When the unbound material was reisolated and incubated again with PHA only a few percentage of it bound, indicating that II-D-3 contains a small amount of glycopeptide material that is incapable of binding to PHA. This inactive material contains sialic acid, galactose, and mannose in amounts roughly proportional to those found in the over-all composition of II-D-3 (Table II). However, the amount of inactive material recovered was so small that precise quantitative determination could not be performed.

In all, six different binding studies were performed with different concentrations of PHA. The amount of glycopeptide bound to PHA was measured and the amount of free PHA and free glycopeptide were calculated from the known amounts of each initially present. The association constant, K = ([glycopeptide bound to PHA]/(free PHA) (free glycopeptide), was then calculated and the average value was 4.7 × 10³ M⁻¹.

Effect of Glycopeptide II-D-3 on Phytohemagglutinin Binding to Lymphocytes—The standard hemagglutination inhibition assay for PHA inhibitory activity tests only the ability of the glycopeptide to inhibit PHA binding to erythrocytes. Previously we have shown that a partially purified glycopeptide preparation from erythrocytes is also capable of inhibiting PHA binding to lymphocytes and of abolishing the mitogenic response induced by PHA (3). As shown in Fig. 7, the glycopeptide II-D-3 is a potent inhibitor of the binding of purified ¹²⁵I-erythroagglutinating PHA to lymphocytes. Using the data in Fig. 7 and knowing the amount of ¹²⁵I-PHA bound by these same lymphocytes at various concentrations of PHA it was possible to calculate an approximate association constant of PHA for II-D-3 (K = 4.5 × 10³ M⁻¹) which agreed very well with the value from the direct binding studies.

Comparison of Phytohemagglutinin Inhibitory Activity of Various Oligosaccharides—The sugar sequence studies (Table III) show that the glycopeptide II-D-3 has an oligosaccharide chain containing the sequence sialic acid → galactose → N-acetylglucosamine with the galactose residue being essential for PHA inhibitory activity. As this is a fairly common sugar sequence in various oligosaccharides, we examined the ability...
of a number of compounds with known structures to inhibit PHA in the standard hemagglutination inhibition assay system. The results are shown in Table V. The glycopeptide II-D-3 had the most potent PHA inhibitory activity of the compounds tested. Both the fetuin and transferrin glycopeptides had considerable inhibitory activity. On the other hand, the oligosaccharides lacto-N-tetraose, lacto-N-neotetraose, and lactosamine, all of which contain the sequence galactose + N-acetylglucosamine, had less than 0.1% the inhibitory activity of II-D-3. These data suggest that the inner sugars of the oligosaccharide chain, particularly the mannose residues, are also involved in the binding of PHA to the oligosaccharide. The simple sugar N-acetylgalactosamine had a slight inhibitory activity at high concentrations but N-acetylgalactosamine and galactose were virtually devoid of activity in this assay system.

Role of Protein Backbone in Phytohemagglutinin Inhibitory Activity of the Glycoprotein—To determine whether the proteolytic action of the trypsin in splitting the glycopeptide from the erythrocyte cell surface was producing a fragment with reduced PHA binding activity compared to the intact site on the cell surface, another technique was employed to solubilize the PHA receptor site in a less "degraded" state. When erythrocyte ghosts were extracted with chloroform-methanol, virtually all of the sialic acid-containing material was solubilized into the aqueous layer in the form of a large molecular weight glycoprotein (Fig. 8). This material, which was totally excluded from Sephadex G-75 (mol wt > 50,000), had high levels of PHA inhibitory activity (64,000 inhibitory units derived from 100 ml of erythrocyte ghosts). Fig. 8 also shows that the usual trypsin procedure when applied to 100 ml of the erythrocyte ghosts liberated only 30% of the sialic acid in the form of lower molecular weight glycopeptide chains.
present bound 5700 cpm.

point was run in duplicate and the lymphocytes with no II-D-3 pellet was counted in a Packard autogamma spectrometer. Each tubes (previously soaked in 5 mg per ml of albumin to reduce non-specific adsorption) to which all components except the cells were added and allowed to incubate for 5 min. The reaction was begun with the addition of the lymphocytes and the incubation continued for 30 min at 37° in a 5% CO₂-95% air atmosphere incubator by which time the binding reaction had reached equilibrium. Five milliliters of 0.9% NaCl-0.01 M NaHCO₃ were added to each tube, the lymphocytes were sedimented and washed with another 5 ml of 0.9% NaCl-0.01 M NaHCO₃, and then the cell pellet was resuspended in a Fackarl autogamma spectrometer. Each point was run in duplicate and the lymphocytes with no II-D-3 present bound 5700 cpm.

**Fig. 7.** Inhibition of 131I-PHA binding to lymphocytes by glycopeptide II-D-3. Lymphocytes (2 × 10⁶) isolated by the method of Boyum (16) were incubated in Eagle's minimum essential medium containing 5 mg per ml of bovine serum albumin, 1.58 µg of 131I-erythroagglutinating PHA (48,500 cpm per µg), and various amounts of glycopeptide II-D-3 as shown in a total volume of 0.4 ml. The binding reactions were carried out in plastic counting tubes (previously soaked in 5 mg per ml of albumin to reduce non-specific 131I-PHA adsorption) to which all components except the cells were added and allowed to incubate for 5 min. The reaction was begun with the addition of the lymphocytes and the incubation continued for 30 min at 37° in a 5% CO₂-95% air atmosphere incubator by which time the binding reaction had reached equilibrium. Five milliliters of 0.9% NaCl-0.01 M NaHCO₃ were added to each tube, the lymphocytes were sedimented and washed with another 5 ml of 0.9% NaCl-0.01 M NaHCO₃, and then the cell pellet was resuspended in a Fackarl autogamma spectrometer. Each point was run in duplicate and the lymphocytes with no II-D-3 present bound 5700 cpm.

**Fig. 8.** Comparison of chloroform-methanol (C-M) extraction and trypsin treatment on releasing PHA inhibitory activity from red blood cell ghosts (RBC ghosts). The chloroform-methanol extraction and analysis of fractions for sialic acid were performed as described under “Experimental Procedure.” The trypsin treatment of ghosts and the aqueous layer from the chloroform-methanol extraction was carried out for 1 hour at 37° after addition of 1 volume of 0.9% NaCl-0.05 M phosphate, pH 7.5, containing 0.25 mg per ml of trypsin to 1 volume of material to be treated. MW, molecular weight; I.U., inhibitory unit.

Glycopeptides with only 3,500 inhibitory units. When the trypsin-treated ghosts were then extracted with chloroform-methanol, the aqueous layer contained all the remaining sialic acid in high molecular weight glycopeptides having only 3,575 inhibitory units. Thus, the trypsin treatment had left behind on the ghosts the majority of their sialic acid but less than 10% of their original chloroform-methanol extractable PHA inhibitory activity. The most logical explanation for the fact that the total activity in the two fractions (trypsin-released plus chloroform-methanol-extracted) was so much less than the total activity in the direct chloroform-methanol extraction of ghosts, was that proteolytic digestion by trypsin could reduce the activity of the receptor site. To test this possibility, the large molecular weight glycopeptides from the direct chloroform-methanol extraction were treated with trypsin and found to be degraded to lower molecular weight glycopeptides (retarded on Sephadex G-75) which retained only 10% of the PHA inhibitory activity or 6,400 inhibitory units. Therefore, it can be concluded that the glycopeptides released from the red cell surface by trypsin do represent fragments of a larger glycoprotein moiety of the surface membrane which when intact has a much greater affinity for binding to PHA. The final product by both routes (trypsin or chloroform-methanol + trypsin) is a small molecular weight glycopeptide of similar activity but the trypsin method releases fragments more selectively so that the active glycopeptides are much less contaminated with inactive sialic acid-containing glycopeptides.

**DISCUSSION**

We have previously demonstrated that trypsin treatment of human erythrocytes releases from the cell membrane glycopeptide fragments which retain their capacity to bind to PHA (3). These glycopeptides have multiple oligosaccharide chains, of which only a portion are capable of binding to PHA (3). By using a combination of alkaline borohydride treatment, Pronase digestion, repeated gel filtration, and DEAE-cellulose column chromatography we have been able to isolate a purified glycopeptide which has potent PHA inhibitory activity. This glycopeptide fraction contains about 25% inactive molecules (i.e., cannot bind to PHA) which have the same gross composition as the active molecules. They must also have a structure very similar to that of the active molecules since the degradation studies on the glycopeptide fraction II-D-3 with β-galactosidase 

Galactose \( \rightarrow \) N-acetylglucosamine 

2. The other branch terminates in sialic acid. Removal of the sialic acid exposes an additional galactose residue which can be removed by β-galactosidase, to expose an additional N-acetylglucosamine residue susceptible to cleavage by β-N-acetylglucosaminidase.

Galactose \( \rightarrow \) galactose \( \rightarrow \) N-acetylglucosamine

3. The inner core of the oligosaccharide after removal of the two termini ([sialic acid, 2 galactose, 2 N-acetylglucosamine residues]) contains 2 mannose residues and 1 N-acetylglucosamine. The fact that neither of the mannose residues had been released by alkaline borohydride treatment suggested that they are not linked O-glycosidically to the serine or threonine residues of the glycopeptide backbone. Further, since both of the mannose residues...
could be removed from the core with $\alpha$-mannosidase, they must be distal to the N-acetylglucosamine residue, which is presumably linked to the asparagine residue of the peptide. The existence of a glycosylamine-type linkage is suggested since the glycopeptide was resistant to alkaline borohydride treatment. However, direct proof that this is the linkage would require isolation of $\beta$ asparaginylglucosamine, and this has not yet been done. These data are consistent with the structure shown in Fig. 9. Note that while the sequence of the two branches of the oligosaccharide chain is established, the attachment of these branches to the inner core remains undetermined. Since the sialic acid-containing chain protects about 85% of the mannose residues from $\alpha$-mannosidase action, approximately 70% of the glycopeptide molecules in II-D-3 must have a structure of the type shown in Fig. 10. The structure of the other 30% of glycopeptide molecules in II-D-3 would be of the type shown in Fig. 10, B and C.

The periodate oxidation studies provide information about the nature of the linkages in the oligosaccharide chain. The galactose residues must be linked either $\beta 1 \rightarrow 3$ or $\beta 1 \rightarrow 4$ to the N-acetylglucosamine residues since the latter are periodate resistant. Since the mannose residues are mostly destroyed by periodate treatment, carbon 3 of the majority of these molecules must not be involved in a linkage. Similarly, since both of the galactose residues were destroyed by periodate, the attachment of sialic acid cannot be through carbon 2 of the galactose residue which is not terminal.

The proposed structure of this glycopeptide from the erythrocyte membrane has many similarities to the glycopeptides reported to occur in plasma proteins, enzymes, and hormones. The trisaccharide sialic acid $\rightarrow$ galactose $\rightarrow$ N-acetylglucosamine occurs in a number of these compounds. Another common feature is the presence of mannose residues in the inner core of the oligosaccharide chain. Finally, a glycosylamine-type linkage between N-acetylglucosamine and an asparagine residue has been a frequent finding in these glycoproteins. The amino acids found in the erythrocyte glycopeptide (aspartic acid, serine, and threonine) are also commonly present near the carbohydrate-protein linkage in serum glycoproteins and it has been suggested that these amino acid residues may act as a code for the enzymes which initiate oligosaccharide chain growth in the peptide backbone.

The biological activity of the oligosaccharide chain is also affected by the nature of the protein backbone. As shown in Fig. 8, trypsin digestion of a high molecular weight glycopeptide results in a 90% loss of PHA inhibitory activity. The most likely explanation for this is that the protein backbone holds the oligosaccharide chains in a favorable conformation. An analogous situation is found in the case of soluble blood group substances. In these high molecular weight glycoproteins the
specific determinants are located in the carbohydrate chains but the protein backbone has a major effect on the reactivity of the molecule. Thus, if these molecules are subjected to proteolytic digestion, a marked loss of biologic activity occurs (24).

During the course of purification of II-D-3 numerous other glycopeptide fractions with PHA inhibitory activity were also separated. In order to determine whether the structures of these active glycopeptides were similar to II-D-3, a few such fractions have been further purified and subjected to structural analysis. The carbohydrate portion of each active glycopeptide examined have in common with glycopeptide II-D-3 a branched oligosaccharide chain with a structure similar to that shown in Fig. 9. In addition some of these glycopeptides contain significant amounts of fucose and an over-all composition consistent with the presence of an additional carbohydrate chain of different structure. Further, these glycopeptides differ in the number and kind of amino acid residues left on the glycopeptide fragments following the Pronase digestion step. These features, combined with the fact that the branched oligosaccharide moiety can contain either 1 or 2 terminal sialic acid residues, most likely account for the size and charge heterogeneity displayed by active glycopeptides.

The finding that the highly purified glycopeptide II-D-3 retains the ability to inhibit PHA binding to lymphocytes suggests that the erythroagglutinating PHA may bind to a similar oligosaccharide on both the lymphocyte and erythrocyte cell surfaces. We are currently attempting to establish this point directly by isolating the PHA receptor site from lymphocyte membranes.

Acknowledgment—We wish to acknowledge the excellent technical assistance of Mrs. Carolyn Noll.

REFERENCES

1. Nespitz, C. K., and Richter, M., Progr. Allergy, 12, 1 (1968).
2. Weber, T., Nordman, C. T., and Grassbeck, R., Scand. J. Haematol., 4, 77 (1967).
3. Kornfeld, S., and Kornfeld, R., Proc. Nat. Acad. Sci. U. S. A., 63, 1439 (1969).
4. Hunter, W. M., in D. M. Weir (Editor), Handbook of experimental immunology, Blackwell Publishing Company, London, 1967, p. 608.
5. Winzler, R. J., Harris, E. D., Pekas, D. J., Johnson, C. A., and Weber, P., Biochemistry, 6, 2195 (1967).
6. Warren, L., J. Biol. Chem., 234, 1971 (1959).
7. Tunis, M., Fed. Proc., 27, 813 (1968).
8. Hodge, J. E., and Hofreiter, B. T., in R. L. Whistler and M. L. Wolfrom (Editors), Methods in carbohydrate chemistry, Vol. 1, Academic Press, New York, 1962, p. 388.
9. Park, J. T., and Johnson, M. J., J. Biol. Chem., 181, 149 (1949).
10. Dische, Z., and Shettles, L. B., J. Biol. Chem., 175, 595 (1948).
11. Reissig, J. L., Strominger, J. L., and Leloir, L. F., J. Biol. Chem., 217, 930 (1955).
12. Li, Y.-T., J. Biol. Chem., 242, 5474 (1967).
13. Jamison, G. A., in H. Peffers (Editor), Proteids of the biological fluids, Vol. 14, American Elsevier Publishing Company, New York, 1966, p. 71.
14. Spho, R. G., J. Biol. Chem., 240, 1603 (1965).
15. Anderson, B., Hoffman, P., and Metee, K., J. Biol. Chem., 240, 156 (1965).
16. Boyum, A., Scand. J. Clin. Lab. Invest., 21, Suppl. 67 (1968).
17. Spho, R. G., J. Biol. Chem., 239, 567 (1964).
18. Kuhn, R., and Baer, N. H., Chem. Ber., 89, 501 (1956).
19. Kuhn, R., and Gauhio, A., Chem. Ber., 66, 618 (1963).
20. Neuberger, A., and Marshall, R. D., in A. Gottschalk (Editor), Glycoproteins, American Elsevier Publishing Company, New York, 1966, p. 275.
21. Sharon, N., Annu. Rev. Biochem., 35, 507 (1966).
22. Eylar, E. H., J. Theor. Biol., 10, 89 (1965).
23. Mark, A. M. S., Donald, A. S. R., Watkins, W. M., and Morgan, W. T. J., Nature, 215, 1345 (1967).
24. Pusztai, A., and Morgan, W. T. J., Biochem. J., 81, 639 (1961).

by guest on March 22, 2020
http://www.jbc.org/
The Structure of a Phytohemagglutinin Receptor Site from Human Erythrocytes
Rosalind Kornfeld and Stuart Kornfeld

*J. Biol. Chem.* 1970, 245:2536-2545.

Access the most updated version of this article at http://www.jbc.org/content/245/10/2536

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/245/10/2536.full.html#ref-list-1