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Rosette formation between human lymphocytes and sheep erythrocytes. Inhibition of rosette formation by specific glycopeptides.

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Research Article

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Rosette Formation between Human Lymphocytes and Sheep Erythrocytes

INHIBITION OF ROSETTE FORMATION BY SPECIFIC GLYCOPEPTIDES

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ABSTRACT Rosette formation with unsensitized sheep erythrocytes is a characteristic of human thymus dependent lymphocytes. Release of glycopeptides from the sheep erythrocyte by trypsin reduces rosette formation. These tryptic glycopeptides inhibit rosette formation by untrypsinized sheep erythrocytes; this suggests that rosetting is mediated by erythrocyte surface glycopeptides. To investigate the molecular nature of this interaction, we examined the abilities of various model compounds to act as haptenic inhibitors of rosette formation. Inhibition is given by glycopeptides bearing oligosaccharide units rich in sialic acid, galactose, *N*-acetylglucosamine, and mannose linked to asparagine residues through glycosylamine bonds. Among compounds tested, fetuin glycopeptide is most effective, but human transferrin glycopeptide and human erythrocyte glycopeptide I also inhibit rosette formation. Other compounds including human erythrocyte glycopeptide II, human IgG glycopeptide, lacto-*N*-neotetraose, 3′- and 6′-sialyllactose show no significant inhibition. Neither sialic acid, galactose, mannose, nor *N*-acetylglucosamine alone inhibits rosette formation. Stepwise degradation of fetuin glycopeptide establishes the galactose residues as important determinants of inhibitory activity.

Fetuin glycopeptide blocks rosette formation when added to a suspension of human lymphocytes and sheep erythrocytes, or when preincubated with human lymphocytes, but not when preincubated with sheep erythrocytes. Studies of the binding of [^{3}H]fetuin glycopeptide to normal human lymphocytes demonstrate 7.5×10^6 saturable binding sites per cell. No saturable binding of this compound to sheep erythrocyte membranes is observed. Compared to normals, lymphocytes from patients with chronic lymphatic leukemia demonstrate decreased fetuin glycopeptide binding with a mean of 0.9×10^6 sites per cell. This decreased binding correlates with the impaired ability of these cells to form rosettes. The data suggest that fetuin glycopeptide inhibits rosette formation by binding to the thymus-dependent cell where competition occurs with sheep erythrocytes for specific lymphocyte surface receptors.

INTRODUCTION

The immune system in higher organisms is composed of many lymphocyte subpopulations. In the human, one subpopulation of lymphocytes interacts in vitro with unsensitized sheep erythrocytes to form structures termed "rosettes" (1–4). Rosettes (Fig. 1) consist of a central lymphocyte surrounded by a cluster of attached sheep erythrocytes. Evidence suggests that the human rosette-forming cell is the thymus-dependent (T) lymphocyte[1] (5, 6) and rosette formation is at present the most commonly used method for identifying this important lymphocyte subpopulation. Indeed, the technique has been widely applied to assess the normal distribution of T lymphocytes, as well as their participation in various pathological states. Despite the usefulness of rosette formation as a clinical tool and its potential importance for further understanding basic mechanisms of human immunity, it remains a poorly understood phenomenon. The present studies were undertaken to investigate the molecular basis for rosette formation.

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[1] *Abbreviations used in this paper:* CLL, chronic lymphatic leukemia. T lymphocyte, thymus-dependent lymphocyte.

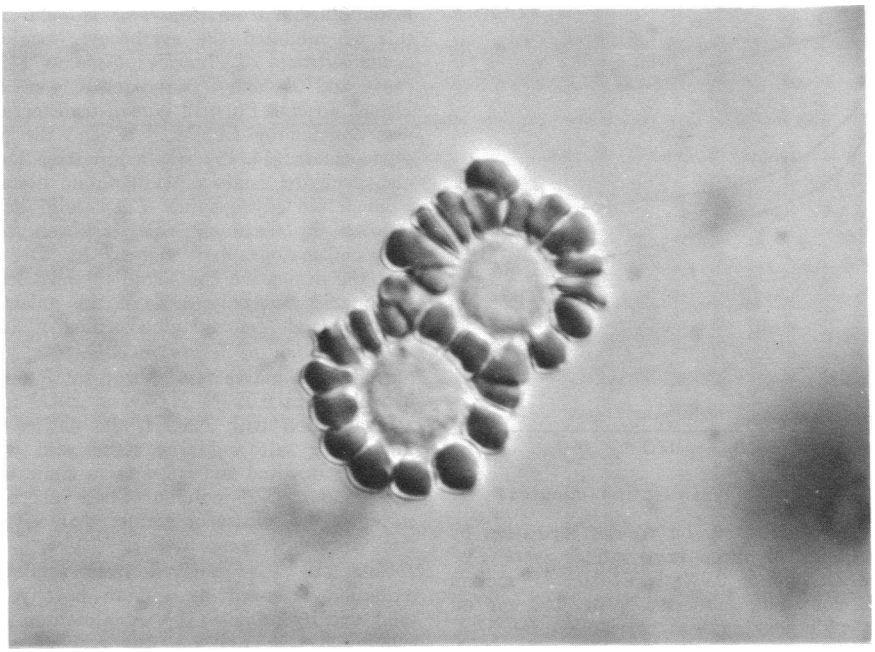


FIGURE 1 Rosette formation between human peripheral blood lymphocytes and sheep erythrocytes. Rosette formation was carried out as described in Methods. Rosettes were photographed using interference phase microscopy (× 1,600).

The findings indicate that rosette formation is mediated through the interaction of sheep erythrocyte surface glycopeptides with receptor sites on the lymphocyte membrane. To examine structural features of this interaction we have studied the effect of model compounds upon rosette formation.

METHODS

Fetuin (B grade), pronase (B grade), and trypsin (A grade), were purchased from Calbiochem, San Diego, Calif. Galactose dehydrogenase from *Pseudomonas fluorescens* (1.1.1.48) and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo. HEM Laboratories, Rockville, Md. provided fetal calf serum, and human AB serum was purchased from Grand Island Biological Co., Grand Island, N. Y. Medium-199 came from Microbiological Associates, Bethesda, Md. Pharmacia Fine Chemicals Inc., Piscataway, N. J. was the source for ficoll and dextran T250 (mol wt 250,000). [^{3}H]Sodium borohydride (700 mCi/mmol) and NCS solubilizer were supplied by Amersham/Searle Corp., Arlington Heights, Ill. Winthrop Laboratories, New York provided sodium diatrizoate (Hypaque sodium, 50% wt/vol), and Hydromix scintillation counting cocktail was purchased from Yorktown Research Inc., S. Hackensack, N. J.

Cell preparation. Venous blood was obtained from healthy young adults. Lymphocytes were isolated from defibrinated whole blood by dextran sedimentation of the erythrocytes followed by isopyknic centrifugation of the leukocyte-rich supernate on a Ficoll-Hypaque mixture (7). Resultant cell preparations consisted of greater than 90% lymphocytes. Lymphocytes from six untreated patients with chronic lymphatic leukemia (CLL) were purified in the same fashion.

Spleens were removed from 250–300-g Walter Reed strain guinea pigs sacrificed by cervical dislocation. Cell suspensions were prepared by pressing the spleens through wire mesh followed by filtration through gauze and isopyknic centrifugation on Ficoll-Hypaque. The resultant cell suspensions contained 75–80% mononuclear cells.

Sheep erythrocytes were obtained weekly from the Walter Reed Army Institute of Research Animal Holding Facility. Blood obtained from 45–80-kg crossbred animals was drawn directly into acid citrate dextrose solution. The erythrocytes were sedimented and washed four times with several volumes of 0.9% NaCl-0.01 M $NaHCO_3$ before use. Erythrocyte membranes were prepared from these cells by lysis at 4°C in 9 vol of distilled water, then washed extensively with 0.01 M Tris, pH 7.4, 0.001 M EDTA (8).

Rosette formation. A modification of the method of Wybran et al. (9) was used for rosette formation. In 12 × 75-mm glass culture tubes (Kimble Products Div., Owens-Illinois, Inc., Toledo, Ohio), 5×10^5 lymphocytes were suspended in 0.2 ml of tissue culture Medium-199 containing 25% heat-inactivated fetal calf serum or pooled human AB serum. All sera were preabsorbed against sheep erythrocytes. To the lymphocyte suspension, 8×10^6 sheep erythrocytes in 0.1 ml 0.9% NaCl-0.01 M $NaHCO_3$ was added, and the mixture was centrifuged at 500 *g* for 5 min. After incubation at 25°C for 60 min, the cell pellet was gently resuspended, and the percentage of lymphocytes forming rosettes was determined by counting 100–200 leukocytes in a microscope hemacytometer chamber. A rosette was defined as a lymphocyte with two or more adherent sheep erythrocytes. For assays of rosette inhibition, test compounds were included in this standard reaction mixture in concentrations as indicated.

Preparation of tryptic glycopeptides from sheep erythrocytes. Sheep erythrocytes were treated with trypsin as described by Winzler et al. (10). Packed washed sheep cells

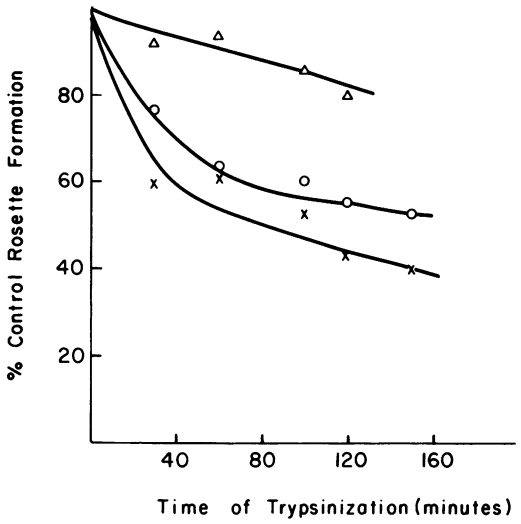


FIGURE 2 Effect of trypsinization on rosette formation by sheep erythrocytes. Washed sheep erythrocytes were suspended in an equal volume of 0.9% NaCl-0.05 M sodium phosphate, pH 7.5, containing either 0.25 or 0.50 mg/ml trypsin. Incubation with shaking was carried out at 37°C for 30–150 min. Cells were washed with bicarbonate-buffered saline three times or until supernate became clear. Parallel incubations without trypsin were used as controls. Washed cells were then used in the standard assay for rosette formation. (△) control incubation; (○) 0.25 mg/ml trypsin; (×) 0.50 mg/ml trypsin.

were suspended in an equal volume of 0.9% NaCl-0.05 M phosphate buffer, pH 7.5, containing 0.25 mg/ml of trypsin. Incubation was carried out at 37°C for 1 h with shaking. A small amount of hemolysis was observed under these conditions. The trypsinized cells were removed by centrifugation, and to the chilled supernate containing the trypsin-released glycopeptides, one-eighth-vol cold 50% trichloroacetic acid (TCA) was added. The resulting precipitate was removed by centrifugation at 15,000 *g* for 15 min at 4°C, and the supernate was neutralized with NaOH. The tryptic glycopeptides were dialyzed exhaustively against distilled water at 4°C and then lyophilized.

Oligosaccharides. The *N*-glycosidically linked glycopeptide of fetuin (fetuin glycopeptide I) [2] was prepared by the method of Spiro and Bhoyroo (11), using extensive pronase digestion of native fetuin, followed by Sephadex G-50 gel filtration. This material eluted from the gel in a position corresponding to a small molecular weight compound of approximately 5,000 daltons. The ratio of sialic acid to hexose in this compound ranged from 1:2.1 to 1:2.6 in good agreement with the predicted ratio for fetuin glycopeptide I (Table I). Human erythrocyte glycopeptides I (8) and II (12) were prepared from outdated bank blood by the methods described by Presant and Kornfeld (12). The purified human erythrocyte glycopeptide I effectively inhibited agglutination of human erythrocytes by *E*-phytohemagglutinin from *Phaseolus vulgaris* (8) while glycopeptide II inhibited the erythrocyte agglutination caused by crude extracts of *Agaricus bisporus* (12). Lacto-*N*-neotetraose and 3'- and 6'-sialyllactose were kindly provided by Dr. K. Joseph. Purified human transferrin glycopeptide (13) was donated by Dr. M. Jett, and the purified human IgG glycopeptide (14, 15) was a gift from Dr. R. Kornfeld, who also provided asialo-agalacto fetuin glycopeptide I produced from fetuin glycopeptide I by mild acid hydrolysis (16) followed by treatment with jackbean β-galactosidase (17). Gas chromatographic analysis by Dr. Kornfeld indicated that the derivative compound retained less than 15% of the sialic acid and only 25% of the galactose but all of the mannose and *N*-acetyl glucosamine of the parent compound.

Degradation of fetuin glycopeptide. Sialic acid was removed from native fetuin or from fetuin glycopeptide I by hydrolysis in 0.05 N H_2SO_4 at 80°C for 1 h (16). After neutralization with NaOH, the native fetuin was dialyzed to remove salt and free sialic acid, while the glycopeptide was desalted and freed from sialic acid by passage over a Sephadex G-25 column. These procedures regularly resulted in the release of greater than 95% of sialic acid residues from these compounds.

Galactose was removed from asialofetuin by periodate oxidation followed by sodium borohydride reduction and mild acid hydrolysis (18). Asialofetuin, 12 mg/ml in 0.05 M sodium acetate, pH 4.5, was reacted with 0.01 M sodium metaperiodate at 4°C for 6 or 24 h in the dark. The reaction was terminated with a 2.5-fold molar excess of ethylene glycol, and the mixture then dialyzed against distilled water. The salt-free material was reduced with 0.15 M sodium borohydride in 0.15 M sodium borate buffer, pH 8.0, for 16 h at 4°C. The pH was adjusted to 5.0 using acetic acid and the preparation again dialyzed. Acetal bonds were cleaved by heating at 80°C for 60 min in 0.05 N H_2SO_4. The sample was neutralized with NaOH, dialyzed, then lyophilized. This procedure resulted in release of approximately 80–90% of galactose residues from asialofetuin.

The *N*-glycosidic glycopeptides were prepared from both asialofetuin and asialo-agalacto-fetuin by pronase digestion and gel filtration as described above.

Preparation of radiolabeled fetuin glycopeptide I. Fetuin glycopeptide I was labeled in the terminal sialic acid residues with tritium using the method of Van Lenten and Ashwell (19). 4–5 μmol of glycopeptide in 0.1 M sodium acetate-0.15 M NaCl, pH 5.6, were reacted with 0.01 M sodium metaperiodate for 10 min at 4°C. A molar excess of ethylene glycol was added, the mixture was concentrated and passed over Sephadex G-25 to separate the glycopeptide from the salt. The glycopeptide in 0.05 M sodium phosphate, pH 7.4, 0.15 M NaCl was treated with 15 mg of sodium borotritide (diluted to a sp act of 14 μci/μmol with sodium borohydride) in 0.01 M NaOH. The reaction was allowed to proceed for 60 min at 25°C with constant stirring; the mixture was then concentrated and passed over Sephadex G-25. The glycopeptide material eluting from the column was pooled and lyophilized, yielding ^{3}H-labeled fetuin glycopeptide of sp act of 20,000–30,000 cpm/nmol.

Binding studies. Lymphocyte-binding studies were performed in 13 × 100-mm disposable glass tubes (Kimble) that had been presoaked in 5 mg/ml bovine serum albumin. For studies of [^{3}H]fetuin glycopeptide I binding to lymphocytes, cells (2×10^6) were incubated at 25°C for 45 min in 0.4 ml of Medium-199 containing 1 mg bovine serum albumin and varying concentrations of radiolabeled glycopeptide. Parallel incubations without cells were carried out to correct for nonspecific binding to the glass tubes. After incuba-

[2] The term "fetuin glycopeptide I" is used in this paper to refer to the *N*-glycosidically linked glycopeptide of fetuin, the structure of which is given in Table I. This designation is used to distinguish this structure from the *O*-glycosidically linked fetuin glycopeptide recently described by Spiro and Bhoyroo (11).

tion, cells were harvested by washing them twice with 5 ml 0.9% NaCl-0.01 M $NaHCO_3$. The cell pellets were dissolved in 0.5 ml NCS, transferred to counting vials containing 10 ml Hydromix, and counted in a Beckman LS-345 Liquid Scintillation System (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

For studies of [^{3}H]fetuin glycopeptide I binding to sheep erythrocyte membranes, 10–200 μg of membrane protein was incubated with varying concentrations of radiolabeled glycopeptide in 15-ml thick-walled glass centrifuge tubes (Corex) that had been presoaked in albumin. After incubation, membranes were washed twice with 5 ml of cold bicarbonate-buffered saline and collected by centrifugation at 45,000 *g* for 15 min. The washed membranes were dissolved in NCS and prepared for liquid scintillation counting as described above.

Chemical determinations. Sialic acid was assayed by the method of Warren (20) after hydrolysis in 1 N HCl for 75 s at 100°C (21). Total hexose was measured by the phenolsulfuric method scaled down to one-fifth volume (22). Galactose was assayed enzymatically using galactose dehydrogenase (23). Protein was determined by the method of Lowry et al. (24) using bovine serum albumin standards.

RESULTS

Rosette formation by human peripheral blood lymphocytes and guinea pig spleen cells. With heat-inactivated, preabsorbed fetal calf serum in the incubation mixture, a mean (±SE) of 57±1% of peripheral blood lymphocytes from normal adults formed rosettes. When pooled human AB serum was used instead of fetal calf serum, a mean (±SE) of 44±2% of normal lymphocytes formed rosettes. Lymphocytes from six patients with CLL were assayed for rosette formation using fetal calf serum in the incubation mixture. Although variability was noted among individuals (range of 0.5–20.0% rosette-forming lymphocytes), none of the patients demonstrated a normal percentage of rosette-forming lymphocytes, and the group mean (±SE) was 10±3%. Cell suspensions from guinea pig spleens were found to contain less than 5% rosette-forming cells.

Effect of trypsinization on rosette formation. When washed, packed sheep erythrocytes were trypsinized as described in the Methods section, glycopeptide fragments were released from the cell surface as indicated by the presence of sialic acid and hexose-containing material in the TCA-soluble fraction. Trypsinization released approximately 2.5 μmol of sialic acid/100 ml of treated packed sheep erythrocytes. Using data derived by Eylar et al. (25), this would represent approximately 12% of the total sheep cell-surface content of sialic acid. Treatment of sheep erythrocytes with trypsin reduces their ability to form rosettes. As illustrated in Fig. 2, the inhibitory effect of trypsin is both concentration and time dependent.

Effect of trypsin-released glycopeptides on rosette formation. We considered that trypsin might release erythrocyte surface receptors for rosette formation, and tested the trypsin-released glycopeptides for their ability to inhibit rosette formation.[3] Fig. 3 illustrates that the tryptic glycopeptides inhibit rosetting in a concentration-dependent fashion, suggesting that rosette formation is mediated by sheep erythrocyte surface glycopeptides. The tryptic glycopeptide at concentrations up to 750 nmol sialic acid/ml had no effect on lymphocyte viability as assessed by trypan blue exclusion.

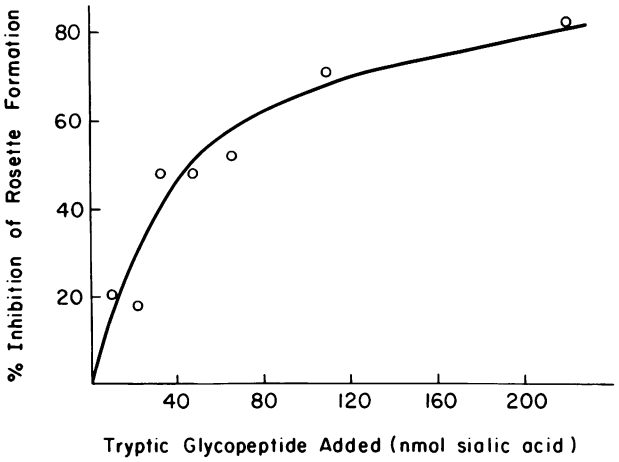


FIGURE 3 Inhibition of rosette formation by tryptic glycopeptides. Sheep erythrocytes were treated with trypsin as described in Methods. After removal of cells by centrifugation, the TCA-soluble tryptic glycopeptides were neutralized, dialyzed against distilled water, and lyophilized. This material was reconstituted with distilled water, then used in the standard assay for rosette formation in the various concentrations shown.

Inhibition of rosette formation by various oligosaccharides. Although the above experiments indicate that sheep erythrocyte tryptic glycopeptides function as cell surface receptors for rosette formation, the limited amounts of inhibitory glycopeptide material obtainable from large quantities of sheep blood restricted the use of this material in further experiments. Therefore, to explore structural features relating to rosette formation, we examined the effects of certain model compounds upon this reaction.

The data in Table I summarize the ability of a number of glycopeptides, oligosaccharides, and simple sugars to act as inhibitors of rosette formation. In addition, Table I illustrates important structural features of the model compounds tested. Fetuin glycopeptide I was the most effective inhibitory compound, but significant in-

[3] A modification of the Thrombo-Wellcotest (Burroughs Wellcome & Co., Greenville, N. C.) for fibrinogen degradation products (26) was employed to assay this preparation for residual proteolytic activity. By this technique, using purified human fibrinogen as substrate, an amount of glycopeptide giving 50% inhibition of rosette formation contained proteolytic activity equivalent to no more than 0.1 μg/ml trypsin.

TABLE I

*Effect of Various Compounds on Rosette Formation**

Compound	Structure	Concentration to give 50% inhibition
		nmol/ml
Fetuin glycopeptide I (ref. 27)	NANA(α 2,3) NANA(α 2,3) NANA(α 2,3) Gal(β 1,4) Gal(β 1,4) Gal(β 1,4) GlcNAc(β 1,2) GlcNAc(β 1,3(4)) GlcNAc(β 1,2(4,6)) Man α 1,2(6)—Man α 1,3—Man 1,4—GlcNAc β 1,4—GlcNAc—Asn	95
Human transferrin glycopeptide (ref. 13)	NANA(2,6) Gal(1,3(4)) GlcNAc(1,3) Man 1,2(4)—Man 1,2(4)—Man 1,2(4,6)—Man 1,3—GlcNAc(1,3(4)) GlcNAc NANA(2,6) Gal(1,3(4)) GlcNAC Asn	190
Human erythrocyte glycopeptide I (ref. 28)	NANA(α 2,6) Gal(β 1,3(4)) GlcNAc(α 1,2) Man α 1,2—Man α 1,?—GlcNAc(β 1,3) Gal(β 1,3 or 4) GlcNAc(β 1,3) Asn	600

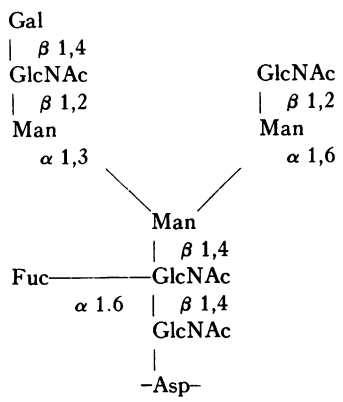
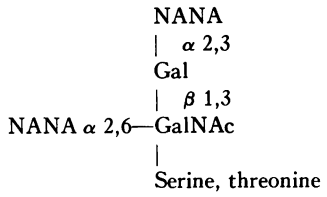
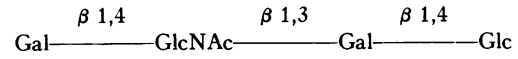
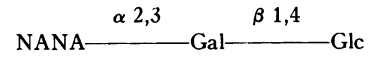
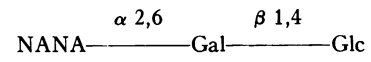
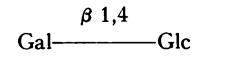
hibition was also provided by human transferrin glycopeptide and human erythrocyte glycopeptide I. In contrast, human IgG glycopeptide inhibited less well and human erythrocyte glycopeptide II was a poor inhibitor, as were the oligosaccharides and simple sugars.

Because of its availability, further studies were carried out with fetuin glycopeptide I. When this compound was hydrolyzed to its component monosaccharides by heating in a boiling water bath for 6 h in the presence of 2 N H_2SO_4 in a sealed vial (18) and then neutralized, its inhibitory activity was abolished. By contrast, the inhibitory activity was not affected by treatment with 0.1 N NaOH under nitrogen for 24 h at 25°C (18), indicating that inhibition was not due to the presence of intact *O*-glycosidically linked fetuin glycopeptide (11) contaminating the fetuin glycopeptide I. Finally, fetuin glycopeptide I, 100 nmol/ml, had no effect upon lymphocyte viability as assessed by trypan blue exclusion.

Fetuin comprises the major α-globulin of fetal calf serum (31). However, the presence of small amounts of fetuin in the standard assay system due to the use of fetal calf serum had no measurable effect upon the results observed. By protein electrophoresis on cellulose acetate in diethyl barbiturate, pH 8.6, the concentration of α-globulin in the fetal calf serum used in these studies was found to be 0.52 g/100 ml. Assuming all the α-globulin to represent fetuin with a mol wt of 45,000 (32), the concentration of fetuin added to the standard rosette assay in fetal calf serum would be 20 nmol/ml. When native fetuin was tested for its ability to inhibit rosette formation, it was found to be relatively ineffective when compared to fetuin glycopeptide I. It required approximately 314 nmol of native fetuin/ml (the equivalent of 942 nmol of *N*-glycosidic chain/ml) to inhibit rosette formation by 50%. Therefore, when the ability of fetuin glycopeptide I to inhibit rosette formation was assessed in parallel incubation mixtures containing either fetal calf serum or human serum, it was found to inhibit equally well in both.

Structural studies with fetuin glycopeptide I. To explore the structural features of fetuin glycopeptide I

TABLE I—(*Continued*)

Compound	Structure	Concentration to give 50% inhibition
		nmol/ml
Human IgG glycopeptide (ref. 14)		>750
Human erythrocyte glycopeptide II (ref. 29)		>3,600
Lacto-*N*-neotetraose (ref. 30)		>3,000
3′-sialyllactose (ref. 30)		>3,000
6′-sialyllactose (ref. 30)		>3,000
Lactose		>3,000
Sialic acid, galactose, mannose, *N*-acetylglucosamine, *N*-acetylgalactosamine		>3,000

* Rosette formation was assayed as described in Methods. Various concentrations from 5 to 3,600 nmol/ml of the compounds listed were added to the standard incubation mixture. The concentration giving 50% inhibition of rosette formation was computed graphically.

important for inhibition of rosette formation, sequential degradation of this compound was undertaken, and the activity of the residual glycopeptide was determined. The results of these experiments are tabulated in Table II. Removal of the terminal sialic acid residues from fetuin glycopeptide I resulted in an increase in rosette inhibition. When both the sialic acid and galactose residues were released, there was a dramatic loss of inhibition. These results were obtained whether the galactose residues were removed enzymatically or by the Smith periodate degradation procedure described in the Methods section.

Preincubation studies. The inhibitory effect of certain oligosaccharides on rosette formation might result from binding to either the lymphocyte or sheep cell or both. Table III shows experiments in which fetuin glycopeptide I was preincubated with either sheep cells or lymphocytes. The cells were washed to remove unbound glycopeptide, then used in rosetting experiments. Preincubation of lymphocytes but not sheep erythrocytes with fetuin glycopeptide I inhibited rosette formation. This information suggests that fetuin glycopeptide I inhibits rosette formation by combining with a site on the lymphocyte surface, thus blocking attachment of the sheep cells.

Binding studies. To test this possibility directly, tri-

TABLE II

*Sequential Degradation of Fetuin Glycopeptide I: Effect of Derivatives on Rosette Formation**

Compound	Concentration to give 50% inhibition	Activity (of control)
	nmol/ml	%
Fetuin glycopeptide I	95	100
Asialo-fetuin glycopeptide I	78	122
Asialo-agalacto-fetuin glycopeptide I	535	18

* Stepwise degradation of fetuin was performed as described in Methods. Fetuin glycopeptide I derivatives were then prepared by extensive pronase digestion. Various concentrations of the derivative compounds were added to the standard assay for rosette formation. The concentration giving 50% inhibition of rosette formation was computed graphically.

tium-labeled fetuin glycopeptide I was prepared and used in binding experiments with normal human lymphocytes. The results of a typical binding study are shown in Fig. 4. The upper panel of this figure shows saturable binding of fetuin glycopeptide I to lymphocytes. In the double reciprocal plot shown below, the y-intercept is a measure of the number of binding sites per cell, whereas the x-intercept is a measure of the binding affinity. In the experiment shown there were 6×10^6 fetuin glycopeptide I-binding sites per lymphocyte, and the apparent association constant, K_a, for fetuin-glycopeptide binding was 8.4×10^4 M^{-1}. The mean ($\pm$SE) from several binding experiments indicates that there are $7.5 \pm 0.5 \times 10^6$ saturable binding sites per lymphocyte with a mean binding affinity given by $K_a = 5.5 \times 10^4$ M^{-1}. When parallel binding studies were performed at 4° and 25°C, identical results were obtained at both temperatures, ruling out the possibility that the observed results represent uptake of glycopeptide rather than binding. When sheep erythrocyte membranes were substituted for the lymphocytes in typical binding reactions, no saturable binding was observed despite use of a wide

TABLE III

*Effect on Rosette Formation of Preincubating Fetuin Glycopeptide I with Sheep Erythrocytes or Lymphocytes**

Experiment	Rosettes per 100 cells counted	% inhibition
1 Control	55	—
2 + fetuin glycopeptide I (287 nmol/ml)	6	89.1
3 Lymphocytes preincubated with fetuin glycopeptide I (287 nmol/ml)	30	45.5
4 Sheep erythrocytes preincubated with fetuin glycopeptide I (287 nmol/ml)	53	3.6

* Lymphocytes (1×10^6) in 0.1 ml Medium-199 or 8×10^6 sheep erythrocytes in 0.1 ml 0.9% NaCl-0.01 M $NaHCO_3$ were incubated with 287 nmol/ml fetuin glycopeptide I for 20 min at 25°C. The cells were washed once with 0.9% NaCl-0.01 M $NaHCO_3$, then used in the standard assay for rosette formation. A control assay contained cells that had not been preincubated. An additional parallel assay contained cells that had not been preincubated plus fetuin glycopeptide I, 287 nmol/ml.

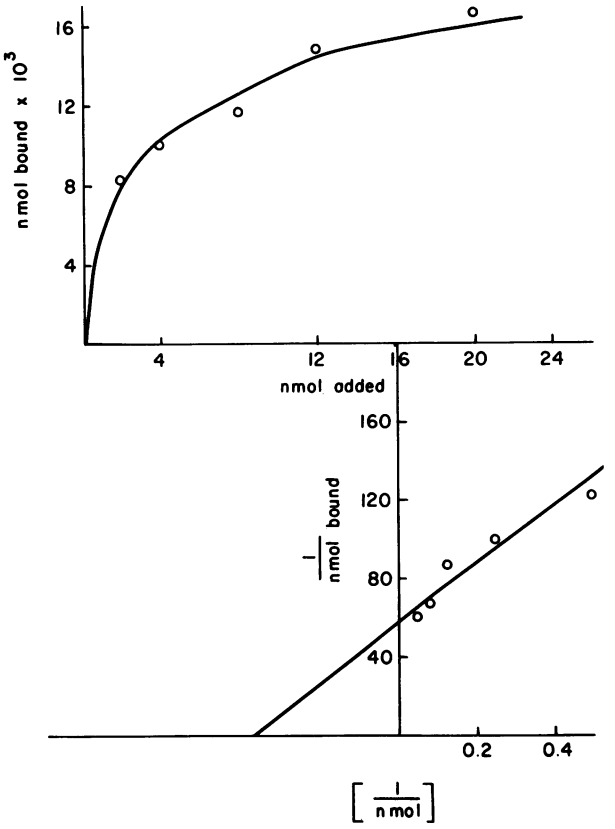


FIGURE 4 Binding of [^{3}H]fetuin glycopeptide I to human peripheral blood lymphocytes. In glass tubes, 2×10^6 human lymphocytes were incubated at 25°C for 45 min in 0.4 ml of Medium-199 containing 1 mg bovine serum albumin and 2–20 nmol [^{3}H]fetuin glycopeptide I. Cells were harvested by washing twice with 0.9% NaCl-0.01 M $NaHCO_3$. The cell pellet was dissolved in 0.5 ml NCS, then taken up in 10 ml Hydromix for counting in a Beckman liquid scintillation spectrometer. Parallel incubation mixtures without cells were carried out to correct for nonspecific binding to the tubes. Data were plotted by the method of Steck and Wallach (33) according to the equation: $1/(F\text{-bound}) = 1/(K \cdot n \cdot C) \cdot 1/F + 1/(n \cdot C)$, where F = concentration of free fetuin glycopeptide I; n = number of fetuin glycopeptide I-binding sites per cell; and C = number of cells. K is the affinity constant of fetuin glycopeptide I.

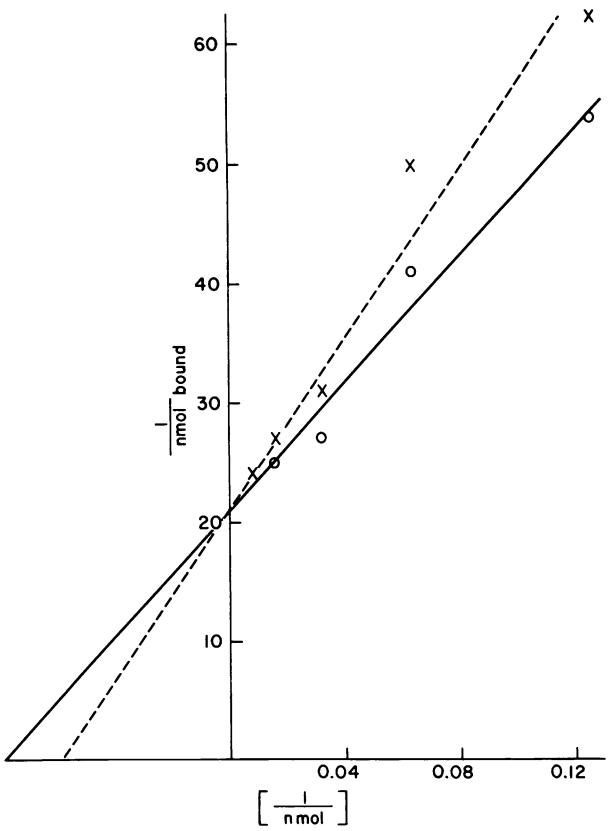


FIGURE 5 Effect of sheep erythrocyte tryptic glycopeptides on binding of [^{3}H]fetuin glycopeptide I to human lymphocytes. As described in the legend to Fig. 4, the binding of radiolabeled fetuin glycopeptide I to human lymphocytes was assessed in the presence and absence of sheep erythrocyte tryptic glycopeptides. Data were plotted by the method of Steck and Wallach (33). (○) control incubation; (×) 375 nmol/ml sheep erythrocyte tryptic glycopeptides.

range of membrane protein concentrations (10–200 μg/0.4 ml incubation mixture). Furthermore, gel filtration experiments[4] demonstrated no interaction between radiolabeled fetuin glycopeptide I and sheep erythrocyte membrane components. These data indicate that there is no specific binding of fetuin glycopeptide I to sheep erythrocyte membranes.

Further studies were carried out to examine the specificity of fetuin glycopeptide I binding. Nonrosetting cells from guinea pig spleens demonstrated no saturable binding of [^{3}H]fetuin glycopeptide I. Lymphocytes from six patients with CLL were assayed for rosette formation, and examined for content of fetuin glycopeptide I-binding sites. Results are given in Table IV. All patients with CLL demonstrate impaired rosette formation compared to normals. Similarly, CLL lymphocytes bear fewer surface receptors for fetuin glycopeptide I than

[4] Boldt, D. H. Unpublished observations.

TABLE IV

Studies of Lymphocytes from Patients with CLL

Patients	Lymphocytes forming rosettes	Molecules [^{3}H]fetuin glycopeptide bound per cell	Absolute lymphocyte count
	%	× 10^6	*cells/mm*^{3}
F. C.	20.0	1.5	19,840
J. D.	18.5	1.7	10,200
L. V.	8.5	0.3	33,110
J. R.	6.5	2.0	25,520
C. V.	4.0	0‡	13,026
M. T.	0.5	0‡	45,900
Normal subjects	57.0	7.5	

* Peripheral venous blood from patients with CLL and from healthy volunteers was processed for lymphocytes as described in the Methods. The percentage of rosette-forming lymphocytes was determined in the standard assay as described. Quantitation of [^{3}H]fetuin glycopeptide-binding was performed as described in the legend to Fig. 4.
‡Only nonsaturable binding was observed.

their normal counterparts. The mean (±SE) number of receptor sites per cell calculated for CLL lymphocytes is $0.9\pm0.4 \times 10^6$ compared with $7.5\pm0.5 \times 10^6$ for normal lymphocytes. The quantity of fetuin glycopeptide I bound per CLL lymphocyte varies considerably among these patients, but for five of the six patients the data in Table IV demonstrate a correlation between rosette formation and fetuin glycopeptide I binding.

Competitive binding studies. These studies were undertaken to investigate the possibility that fetuin glycopeptide I and the sheep erythrocyte surface structure responsible for rosette formation might bind to the same lymphocyte receptor. Results of a typical study are illustrated in Fig. 5. Binding of radiolabeled fetuin glycopeptide I to lymphocytes was competitively inhibited in the presence of sheep erythrocyte tryptic glycopeptides.

DISCUSSION

These studies indicate that rosette formation between human lymphocytes and sheep erythrocytes is mediated through the interaction of trypsin-sensitive sheep cell-surface glycoproteins with the lymphocyte membrane. Glycopeptide material solubilized from the sheep cell surface by trypsin effectively inhibits rosette formation. Because the quantity of inhibitory material retrievable from large quantities of sheep blood was insufficient for extensive studies, we have used model compounds in haptene inhibition experiments to examine the structural nature of the sheep erythrocyte glycopeptides that participate in rosette formation.

The data in Table I demonstrate several important points. First, compounds that inhibit rosette formation share certain structural features. Thus the basic structure sialic acid → galactose → *N*-acetylglucosamine

linked to a core of mannose residues is present in fetuin glycopeptide I, human transferrin glycopeptide, and human erythrocyte glycopeptide I. Second, the potency of the various inhibitors seems to be related to their content of oligosaccharide chains with this basic structure. Thus, fetuin glycopeptide I with three of these chains per molecule is a better inhibitor than the transferrin glycopeptide and the human erythrocyte glycopeptide I which contain only two (Table I). The difference in the inhibitory activity of these latter two glycopeptides may relate to the differences in their core structures.

Further evidence supporting the importance of the core mannose residues for inhibition of rosette formation is derived from a consideration of the data in Table I for several different oligosaccharides. Lacto-*N*-neotetraose and 3′- and 6′-sialyllactose share certain structural sequences with the oligosaccharide chains in fetuin, transferrin, and human erythrocyte glycopeptide I, but lack the mannose core. Lacto-*N*-neotetraose has a galactose → *N*-acetylglucosamine linkage, and 3′- and 6′-sialyllactose have sialic acid linked to galactose. The fact that none of these three compounds shows significant rosette inhibition suggests that these sequences alone are not sufficient structural determinants for this inhibition. Since these compounds lack core mannose residues, these data also suggest that the mannose core may be an important determinant for inhibition.

Further structural information has been derived from the sequential degradation of fetuin glycopeptide I (Table II), the most potent inhibitor of rosette formation that we studied. Cleavage of the terminal sialic acid residues from the three oligosaccharide chains of this molecule resulted in a consistent increase in its ability to inhibit rosette formation. This finding is of interest in light of the fact that several investigators have noted enhancement of rosette formation by human lymphocytes when the sheep erythrocytes used had been pretreated with neuraminidase (34–36).

Removal of the terminal sialic acid and galactose residues from fetuin glycopeptide I abolishes the effectiveness of this compound as an inhibitor of rosette formation. These data suggest that among the key determinants for inhibition of rosette formation by fetuin glycopeptide I are the galactose residues in the outer chains.

Although the galactose residues of fetuin glycopeptide I are of major importance for inhibition of rosette formation, galactose and other simple sugars are ineffective inhibitors when added alone (Table I). Similar results have been noted with respect to the receptor sites for plant lectins (8, 37). Furthermore, when fetuin glycopeptide I is hydrolyzed to its component monosaccharides, its inhibitory effect on rosette formation is lost.

Summarizing these structural studies, we conclude that compounds containing the basic structure sialic acid → galactose → *N*-acetylglucosamine → $(\text{mannose})_n$ are effective inhibitors of rosette formation. The galactose residues are important determinants of this inhibition.

Indirect evidence for the presence on the sheep erythrocyte surface of oligosaccharide chains structurally similar to fetuin glycopeptide I comes from recent work of Sela et al. (38). Using affinity chromatography on fetuin covalently attached to Sepharose to fractionate sera from a variety of animal sources, these workers isolated antibodies which appeared to recognize the specific oligosaccharide sequences of fetuin. These purified immunoglobulin fractions agglutinated erythrocytes from sheep and some other mammalian species. In addition, Fletcher and Woolfolk (39) have isolated a glycoprotein antigen from sheep erythrocytes which contains molar ratios of carbohydrates similar to those ratios in fetuin. Our data (Fig. 5) which illustrate competitive inhibition of radiolabeled fetuin glycopeptide I binding by the sheep erythrocyte tryptic glycopeptides suggest that certain structures in both preparations can bind to the same lymphocyte surface receptor. It therefore seems likely that a structure similar to fetuin glycopeptide I is present on the sheep erythrocyte surface and may take part in rosette formation. However, the possibility remains that steric hindrance or surface charge modification may contribute to the observed effects of fetuin glycopeptide I and structurally related compounds in our system.

The preincubation studies (Table III) and the binding studies with tritiated fetuin glycopeptide I (Fig. 4) indicate that compounds with this basic structure are capable of binding to the human lymphocyte surface. There are approximately 7.5×10^6 binding sites per lymphocyte for fetuin glycopeptide I and the binding affinity, K_a, is $5.5 \times 10^4\ M^{-1}$. This affinity is in accord with the fragile nature of rosettes which tend to dissociate easily. The inhibitory constant, K_i, for the inhibition of rosette formation by fetuin glycopeptide I, is $1.3 \times 10^4\ M^{-1}$. This value is in agreement with the association constant for fetuin binding, indicating that the inhibition of rosette formation by fetuin glycopeptide I is closely related to its ability to bind to the lymphocyte surface. The latter point is strengthened by our inability to demonstrate significant interaction between fetuin glycopeptide I and sheep erythrocyte membranes.

To examine the specificity of the fetuin glycopeptide-lymphocyte interaction and its relationship to rosette formation, we studied the ability of nonrosetting lymphocyte populations to bind radiolabeled fetuin glycopeptide I. Thus mononuclear cells from guinea pig spleens fail to rosette and demonstrate no saturable binding of fetuin glycopeptide I.

Our data on a group of patients with CLL emphasize that the peripheral blood lymphocytes in this disorder are heterogeneous, containing 0.5–20% rosette-forming cells. Similar results have been noted by others (38–42). As shown in Table IV, there is no apparent relationship between the percentage of CLL lymphocytes that form rosettes and the absolute lymphocyte counts of these patients. The results of fetuin glycopeptide I-binding studies in five of the six patients (Table IV) demonstrate a correlation between rosette formation by the CLL lymphocytes and the ability of these cells to bind radiolabeled glycopeptide. In two of the patients (F. C. and J. D.), 15–20% of peripheral blood lymphocytes form rosettes, and these cells bind 1.5 and 1.7 $\times 10^6$ molecules per cell of fetuin glycopeptide I, respectively. By contrast, lymphocytes from patients M. T., C. V., and L. V. contain 0.5, 4.0, and 8.5% rosette-forming cells, respectively. Cells from two of these patients (M. T. and C. V.) fail to demonstrate saturable binding of fetuin glycopeptide I, while lymphocytes from L. V. bind only 300,000 molecules per cell.

The data from the binding studies using CLL lymphocytes and guinea pig spleen cells suggest that fetuin glycopeptide I binds preferentially to human rosette-forming lymphocytes. The ability to bind this compound may thus represent a specific T-cell function. Furthermore, the data on rosette inhibition and the competitive binding studies (Fig. 5) provide the possibility that fetuin glycopeptide I is bound to the lymphocyte via the same surface receptor sites which combine with sheep erythrocytes during rosette formation.

Yachnin has recently demonstrated that fetuin is able to inhibit in vitro human lymphocyte transformation in response to stimulation by various plant mitogens, antithymocyte antiserum, and allogeneic cells (43). Furthermore, Murgita and Tomasi have reported that α-fetoprotein added in vitro to murine lymphoid cells causes suppression of specific murine T-cell-dependent functions such as allogeneic and mitogen-induced lymphocyte transformation (44). In addition this compound can suppress development of primary and secondary antibody production by the lymphoid cells of fetal and neonatal mice (45), perhaps through an effect on T-cell intermediates (45). These observations suggest that lymphocyte receptor sites for fetuin and related fetal proteins such as α-fetoprotein serve important immunoregulatory functions in vivo. Additional experiments will be required to further investigate these intriguing possibilities.

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