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Antibodies Reactive with Cell Surface Carbohydrates

(membrane receptors/lymphocyte stimulation/immunoglobulins/affinity chromatography)

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ABSTRACT **Normal and immune sera from various animal species were fractionated on columns of Sepharose covalently coupled with the glycoprotein fetuin. Elution of the material bound to fetuin yielded low but reproducible amounts of protein, ranging from 0.02 to 0.2% of the protein mass of the input sera. This material has been identified by immunoelectrophoresis in agar and by zone electrophoresis on cellulose acetate as immunoglobulin. The Ig fractions bound and agglutinated erythrocytes of various species, and also bound to cells from various mouse tissues including heart, kidney, thymus, and spleen. In all cases, the binding was inhibited by glycoproteins such as fetuin and thyroglobulin, by a glycopeptide isolated from fetuin, and by some bacterial lipopolysaccharides. When the binding of these Ig fractions to mouse splenocytes was tested in the presence of 17 saccharides, no inhibition of binding was observed except by sialic acid, D-galactose, *N*-acetyl-D-glucosamine, and D-mannose, all of which showed partial inhibition. Inasmuch as these four saccharides are present on the carbohydrate moiety of fetuin, the results suggest that the isolated material is a carbohydrate-specific Ig (CS-Ig) fraction of serum capable of binding to the carbohydrate portion of cell surface receptors and glycoproteins. When bound to lymphocytes, these CS-Ig molecules induced redistribution (patching and capping) of cell surface receptors. Moreover, the CS-Ig fractions from chicken and rabbit sera were weakly mitogenic for mouse splenic lymphocytes. CS-Ig fractions are useful new reagents for studying glycoproteins and the interactions and activities of cell surface carbohydrates.**

Heterosaccharide structures on cell surfaces are known to play a role in a wide variety of phenomena including cellular interactions and the initial events of cell proliferation (1). Adventitious carbohydrate-binding molecules, such as plant lectins (2), have been particularly useful in the quantitation of membrane receptors on mammalian cells, in studies of the redistribution of these receptors, and in provoking resting cells to undergo blast transformation and mitosis. To date, however, only one lectin, concanavalin A (Con A), has been fully characterized in terms of its structure (3) and binding specificity (4). A successful search for other carbohydrate-binding proteins, with well-characterized structures and binding specificities and that are amenable to chemical alteration, would be of great usefulness in analyses of the cell surface.

The existence of "natural" antibodies to carbohydrate components has been most clearly demonstrated in the classic studies of antibodies directed against blood group antigens (5). More recently, various lines of evidence have been accumulated to suggest that other antibodies directed at carbohydrate components of the cell membrane might be detected and isolated (6, 7). Such studies on anti-carbohydrate antibodies have been hampered, however, by difficulty in eliciting specific antibody responses to saccharide moieties of glycoproteins (8). This difficulty may be due to the obvious similarity between the saccharide structures appearing in unrelated serum glycoproteins and in cell surface receptors (Fig. 1) (9–11), rendering immunized animals tolerant to the antigenic determinants.

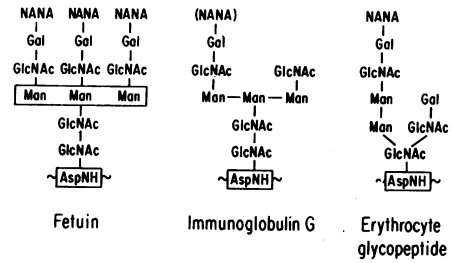

FIG. 1. Proposed structures for the carbohydrate moieties of glycoproteins fetuin (9) and immunoglobulin G (10), and of a glycopeptide isolated from erythrocytes (11). NANA is *N*-acetylneuraminic acid.

We have taken an alternative approach to the isolation of anti-carbohydrate antibodies specific for such structures. In this paper, we describe a procedure for the isolation from normal and immune sera a specific fraction that is bound to the glycoprotein fetuin immobilized on Sepharose columns. This material has been identified as an immunoglobulin with a binding specificity for certain carbohydrates. The biological and structural properties of this carbohydrate-specific Ig (CS-Ig) fraction allow new approaches to the study of cell surface carbohydrates.

MATERIALS AND METHODS

Preparation of Affinity Chromatography Adsorbents. Sepharose 4B (50 ml) was activated with CNBr (12) and thoroughly washed with 0.1 N sodium bicarbonate buffer, pH 8.6. Fetuin (400 mg) was dissolved in 50 ml of 0.1 N sodium acetate buffer, pH 5.5, and the solution was boiled at 100° for 5 min. The material was then dialyzed against 0.1 N sodium bicarbonate, pH 8.6, and added to the CNBr-activated Sepharose. The mixture was gently stirred overnight at 4°, 2-aminoethanol was added to a final concentration of 0.5 M, and the mixture was restirred for another 2 hr. The Sepharose beads were then washed with 0.05 M glycine·HCl, 0.5 M NaCl, pH 3.0, and finally equilibrated with phosphate-buffered saline (pH 7.4, 8.00 g of NaCl, 0.20 g of KCl, 0.20 g of KH_2PO_4, 0.15 g of Na_2HPO_4 per liter). The efficiency of coupling heat-denatured fetuin to the Sepharose was 85–90%. Similar procedures were used to couple porcine thyroglobulin and bovine serum albumin.

Isolation of the CS-Ig Fractions. Normal and immune sera were applied in 100 ml batches onto a Sepharose-fetuin column

Abbreviations: Ig, immunoglobulin; CS-Ig, carbohydrate-specific immunoglobulin; Con A, concanavalin A.

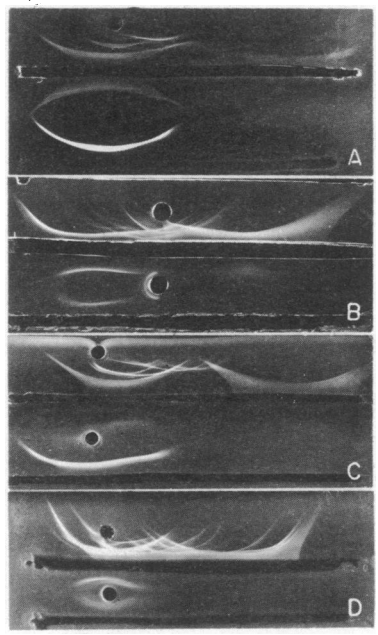

FIG. 2. Representative immunoelectrophoresis patterns of input serum and CS-Ig fractions. (A) upper well: rabbit serum; lower well: CS-Ig (rabbit); upper trough: goat anti-rabbit serum; lower trough: goat anti-rabbit immunoglobulin. (B) upper well: human serum; lower well: CS-Ig (human); upper trough: goat anti-human serum; lower trough: rabbit anti-human IgG. (C) upper well: dog serum; lower well: CS-Ig (dog); upper trough: rabbit anti-dog serum; lower trough: rabbit anti-dog IgG. (D) upper well: turkey serum; lower well: CS-Ig (turkey); upper trough; rabbit anti-turkey serum; lower trough: rabbit anti-turkey immunoglobulin.

(50 ml total volume) at room temperature at a slow flow rate of 10–12 ml/hr. The unbound serum proteins were eluted with phosphate-buffered saline and the column was washed extensively until the absorbance of the effluent at 280 nm was below 0.005. The fetuin-bound material was then eluted with 0.05 M glycine·HCl, 0.5 M NaCl, pH 3.0. The eluted material was immediately readjusted to pH 7.0 and then concentrated by pressure dialysis in Amicon cells with a Diaflo XM-50 membrane.

TABLE 1. *Fractionation of turkey serum on various adsorbents coupled to Sepharose 4B*

Assay	Adsorbent: Sepharose 4B-fetuin	Sepharose 4B-thyroglobulin	Sepharose 4B-bovine serum albumin	Sepharose 4B†
Yield of isolated protein*	6 mg	4 mg	<0.4 mg	<0.2 mg
Identification of isolated protein as Ig	Yes	Yes	No	No
Agglutination activity for mouse erythrocytes	Yes	Yes	No	No

* The reported yield is based on fractionation of 100 ml of turkey serum on each of the respective Sepharose 4B columns.

† The Sepharose 4B beads were activated with CNBr, washed, and used without any adsorbent protein.

Binding of ^{125}I-labeled CS-Ig (dog) to Cells. The carbohydrate-specific Ig fraction obtained from dog serum, CS-Ig (dog), was labeled with ^{125}I by the chloramine-T method (13) (specific activity, 2.2×10^7 cpm/mg). Iodination of CS-Ig (dog) did not alter its binding properties inasmuch as the binding of ^{125}I-labeled CS-Ig (dog) to mouse spleen cells could be inhibited competitively in a linear fashion by unlabeled CS-Ig (dog). For binding experiments, spleen cells from NCS mice (Rockefeller University, New York) were incubated with ^{125}I-labeled CS-Ig (dog) (10 μg/ml) in the presence and absence of inhibitors. The concentration of lymphocytes was 1×10^7 cells per ml in a final volume of 1 ml. After 30 min at room temperature, the cells were washed three times by centrifugation and resuspension, transferred to fresh tubes, and subjected to gamma counting.

The inhibitors used in this study were from commercially available sources. The glycopeptide inhibitor from fetuin was prepared by digestion with Pronase followed by ion-exchange chromatography on Dowex-50 (Bio-Rad AG 50W-X4) and gel filtration on Sephadex G-25. Of the fractions from this gel filtration column, only the second major fraction had inhibitory activity for the binding of ^{125}I-labeled CS-Ig (dog) to spleen cells. This fraction was designated the fetuin glycopeptide.

Serum Sources and Assay Procedures. Antisera against fetuin were raised in rabbits by intramuscular injection in complete Freund's adjuvant. All other normal and immune sera were obtained commercially. Immunoelectrophoresis and gel electrophoresis techniques have been described previously (14, 15). Agglutination assays were performed by serial dilution of the CS-Ig fractions (1 mg/ml) in plastic tissue culture plates. After 30 min at room temperature, agglutination was scored on a scale of (−) to (++++) based on the highest dilution showing agglutination: (−) no agglutination at 1 mg/ml; (+) 1:4 dilution, (++) 1:16 dilution; (+++) 1:64 dilution; (++++) 1:256 dilution. Mitogenesis assays and fluorescence studies of the binding of CS-Ig fractions to cell surface receptors were performed as described previously (16, 17).

RESULTS

Isolation of CS-Ig Fractions by Affinity Chromatography. When normal or immune sera were fractionated on a column of Sepharose-fetuin, a small but reproducible amount of protein material was bound to the column. Addition of 0.05 M glycine·HCl, 0.5 M NaCl, pH 3.0, resulted in the elution of this bound material, designated hereafter as CS-Ig. The amount of CS-Ig material and the elution profile depended on the source of the sera used, but these parameters were constant for several runs if the same serum was used.

Normal dog and turkey sera yielded the largest amount of CS-Ig (5–6 mg/100 ml), while normal human, chicken, and rabbit sera yielded the least (1.5–2 mg/100 ml). Pig, goat, and horse sera yielded 2–3 mg of CS-Ig. Sera from rabbits immunized with mouse lymphocytes did not give a higher yield of CS-Ig protein than normal rabbit sera. In general, the column-bound fraction from various sources ranged from 0.02 to 0.2% of the total protein loaded on the column, assuming the protein concentration of various sera used was 2.5–7 g/100 ml. Less than 0.5 mg of protein could be obtained when a

corresponding amount of calf serum was fractionated. Although the nature of this minute amount of material could not be identified, preliminary immunoelectrophoresis experiments indicated that it did not correspond to the Ig-like material obtained from other serum sources.

In a series of control experiments, 100 ml aliquots of normal turkey serum were fractionated on similar columns differing only in the protein adsorbent coupled to the Sepharose. Comparable yields of CS-Ig material were obtained from Sepharose-fetuin and Sepharose-thyroglobulin columns. In contrast, when the sera were fractionated on Sepharose columns without any glycoprotein (Sepharose-bovine serum albumin and CNBr-activated Sepharose), only minute traces of protein were bound (Table 1). These data suggest that the CS-Ig material of the fractionated serum were bound to the glycoproteins, probably to their carbohydrate moieties.

Characterization of the CS-Ig Fractions. To assess the purity and identity of the serum fraction bound to the Sepharose-fetuin columns, the material was subjected to immunoelectrophoresis and the patterns were compared with those of the input serum. Representative patterns obtained with materials from rabbit, human, dog, and turkey are shown in Fig. 2. In each case, the column-bound material yielded a precipitin arc with both antiserum to whole serum and antiserum to Ig at a position corresponding to the mobility of serum IgG. In some preparations, a faint additional line of precipitate was found at the position of serum albumin. This more negatively charged contaminant could be eliminated by rechromatography of the CS-Ig on the Sepharose-fetuin column.

Although the immunoelectrophoretic patterns of the CS-Ig fractions lacked a precipitin arc at a position corresponding to serum IgM, sedimentation velocity experiments with CS-Ig (dog) showed two species, one of which accounted for about 50% of the material and had a sedimentation coefficient of 7 S. The sedimentation coefficient of the other species was 19 S, suggesting that it represented IgM molecules. In addition, gel electrophoresis experiments (15) showed that CS-Ig fractions from rabbit and chicken sera had mobilities corresponding to those of normal rabbit and chicken immunoglobulin standards. These physico-chemical data strongly support the assignment of the CS-Ig fraction as an immunoglobulin.

Binding Properties of the CS-Ig Fractions. The purified CS-Ig proteins were found to bind cells of different tissues from the mouse, including the heart, kidney, thymus, and spleen. The specificity of the binding was studied using ^{125}I-labeled CS-Ig (dog) and mouse spleen cells in the presence of various inhibitors. The data (Table 2) indicate that fetuin and thyroglobulin inhibited the binding to an extent of 20–30%. A marked inhibition of binding (70%) was observed with the fetuin glycopeptide. In addition, it was found that lipopolysaccharides from *Salmonella typhosa*, *Shigella flexneri*, and *Salmonella enteritidis* were good inhibitors of the binding of CS-Ig (dog) to cells. Finally, the striking observation was made that amongst all the mono- and oligosaccharides tested, only sialic acid, D-galactose, *N*-acetyl-D-glucosamine, and D-mannose showed any significant inhibitory activity (20–30%). Since these sugars are present on the carbohydrate branch of fetuin and thyroglobulin, these observations strongly suggest that the CS-Ig material is specific for saccharide structures composed of these sugars. The fact that a comparable amount (30%) of ^{125}I-labeled CS-Ig (dog) bound on Sepharose-fetuin columns can be eluted by a mixture of these simple sugars is in accord with this conclusion.

TABLE 2. *Inhibition of the binding of ^{125}I-labeled CS-Ig (dog) to mouse splenocytes*

Inhibitor*	^{125}I-labeled CS-Ig (dog) bound to cells (cpm)	% Inhibition
Phosphate-buffered saline	9,600	—
Saccharides		
D-Glucose	9,370	2
D-Galactose	6,910	28
L-Fucose	9,410	2
D-Mannose	7,390	23
N-Acetyl-D-glucosamine	6,720	30
N-Acetyl-D-galactosamine	11,000	0
α-Methyl-D-mannoside	9,700	0
N-Acetylneuraminic acid	7,400	23
D-Trehalose	8,400	12
D-Maltose	9,700	0
β-D-Lactose	8,930	7
D-Melibiose	10,200	0
D-Cellobiose	8,650	9
D-Raffinose	11,000	0
D-Melezitose	13,000	0
Dextran 80	9,100	5
Glycogen	13,000	0
Glycoproteins		
Fetuin	6,970	28
Fetuin-glycopeptide	3,000	70
Ovomucoid	13,000	0
Ovalbumin	12,000	0
Thyroglobulin	7,690	20
Transferrin	9,050	6
Lipopolysaccharides		
Escherichia coli	9,730	0
Salmonella abortus	8,940	7
Salmonella typhimurium	9,850	0
Salmonella typhosa	4,630	52
Shigella flexneri	3,660	62
Salmonella enteritidis	4,220	56

* Inhibitors were tested at a final concentration of 0.02 M for the mono- and oligosaccharides and 2.5 mg/ml for the polysaccharides, glycoproteins, and lipopolysaccharides.

The CS-Ig fractions purified from the various sera were also tested for their agglutinating activity for erythrocytes from different species (Table 3). Whereas the unfractionated sera had no agglutinating activity, virtually all purified CS-Ig fractions from the different species agglutinated one or more of the erythrocyte types tested. In no case, however, did the CS-Ig of one species agglutinate the erythrocytes of the same species.

We also found that antibodies directed against fetuin, which are known to react with the protein portion of this glycoprotein (8), did not agglutinate any of the erythrocytes. These results suggest that the agglutination activity of the CS-Ig fractions is due to the saccharide-specific binding of these molecules to cell surface carbohydrates.

Interaction of CS-Ig with Lymphoid Cells. When CS-Ig fractions were bound to mouse splenic lymphocytes, they in-

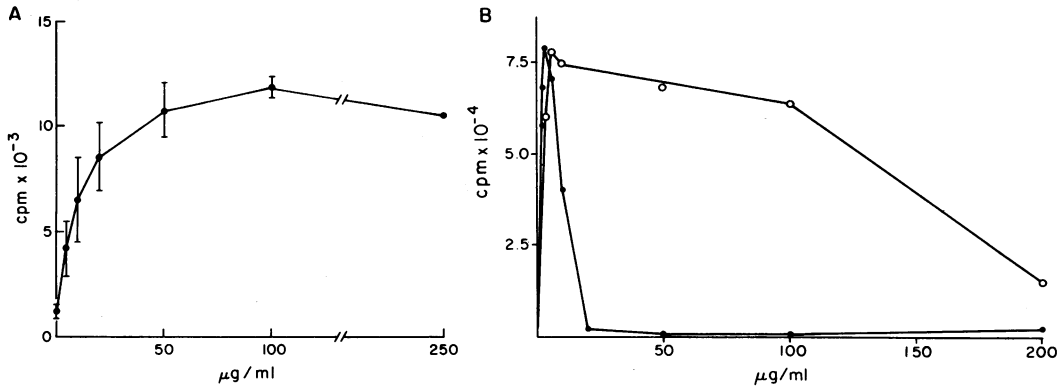

FIG. 3. Dose–response curve of the incorporation of [^{3}H]thymidine in the stimulation of mouse spleen cells by (A) CS-Ig (chicken) and (B) Con A (●) and succinyl-Con A (○).

duced patch and cap formation of the surface receptors, as reported for other exogenous ligands (17). In addition, it was found that the CS-Ig from normal chicken serum and from sera of rabbits immunized with mouse lymphocytes showed mitogenic activity for mouse spleen cells. The dose–response curve for the stimulation of mouse lymphocytes by CS-Ig (chicken) is shown in Fig. 3A. Control experiments showed that the weak but reproducible response of lymphocytes to CS-Ig was not due to antigenic stimulation, inasmuch as normal chicken IgG did not stimulate. Moreover, the mitogenic activation of lymphocytes by CS-Ig (chicken) was inhibited by fetuin or thyroglobulin at inhibitor concentrations of about 250 μg/ml.

The response of mouse splenocytes to CS-Ig showed maximal stimulation at concentrations of 50–100 μg/ml. Furthermore, this response did not decrease at concentrations as high as 200 μg/ml. This resembles the stimulation of mouse splenocytes by divalent succinyl-Con A but not that of native tetrameric Con A (Fig. 3B) (18).

DISCUSSION

From various lines of evidence (19, 20), it has been proposed that the receptors interacting with mitogenic reagents such as lectins and sodium metaperiodate to induce lymphocyte transformation have structures resembling the carbohydrate side chains of certain glycoproteins (Fig. 1) (9–11). Such observations have prompted us to take a direct approach in the search for serum proteins that bind specifically to these saccharide structures. The method described in the present report is a general one that can be used with a variety of prosthetic groups on proteins and with ligands from various species and tissue sources.

With the glycoprotein fetuin as an adsorbent in affinity chromatography, a small amount (0.02–0.2% of the input serum) of protein from both normal and immune sources can be isolated; this material consists of Ig molecules specific for saccharides. Only calf serum failed to yield any protein bound to Sepharose-fetuin. Inasmuch as fetuin is found in both fetal and adult calf serum, it is possible that fetuin-binding proteins in calf sera are already complexed with the glycoprotein and therefore would not be bound to the affinity column.

The serum fraction bound to Sepharose-fetuin was established as an immunoglobulin by immunological (Fig. 2) and physico-chemical techniques. The carbohydrate-binding specificity of these Ig fractions was suggested by the observation that ^{125}I-labeled CS-Ig (dog) bound on Sepharose-fetuin columns could be partially eluted by a mixture of D-galactose and *N*-acetyl-D-glucosamine. In addition, it was found that comparable yields of column-bound material were obtained by fractionating sera on Sepharose-fetuin and Sepharose-thyroglobulin columns, but only minute traces of protein were found when Sepharose-bovine serum albumin or CNBr-activated Sepharose were used (Table 1). We have, therefore, tentatively designated these materials as carbohydrate-specific Ig (CS-Ig) fractions.

Several other lines of evidence argue for the carbohydrate-binding specificity of these CS-Ig fractions (Table 2): (*1*) The partial but significant inhibition of the binding of CS-Ig to mouse spleen cells by four monosaccharides which compose the main part of the carbohydrate moiety of fetuin. Among the 17 mono- and oligosaccharides tested, only sialic acid, D-galactose, *N*-acetyl-D-glucosamine, and D-mannose showed any inhibition of CS-Ig binding to the cells; (*2*) The moderate inhibition of CS-Ig binding to cells by fetuin (28%) was in-

TABLE 3. *Agglutination of erythrocytes from different species by various carbohydrate-specific immunoglobulin fractions*

Carbohydrate-specific Ig fractions	Erythrocytes							
	Rabbit	Pig	Horse	Sheep	Calf	Human	Chicken	Mouse
Rabbit anti-mouse lymphocytes	−	−	−	++	−	−	−	+
Rabbit	−	−	−	+	−	−	−	−
Pig	+	−	++	+	−	−	−	++
Goat	++	−	++	−	−	−	−	+
Horse	+	−	−	−	−	−	−	++
Dog	+++	++	−	−	−	−	−	+++
Human	+	−	−	−	−	−	−	−
Chicken	++	++++	−	−	−	−	−	−
Turkey	+++	++	−	−	−	−	−	++

creased to 70% when a glycopeptide derived from fetuin by Pronase treatment was used as an inhibitor; (3) lipopolysaccharides from certain bacterial sources strongly inhibited the binding of CS-Ig to cells. If any similarity between lipopolysaccharides and fetuin exists, it must reside in the saccharide structures of the two molecules.

Based on these chemical and biological experiments, the isolation of CS-Ig fractions provides a strong indication for the existence in normal sera of antibodies capable of binding to the carbohydrates of various glycoproteins, in addition to the antibodies directed against blood group substances (21). It is known that sera of various animals contain "natural" antibodies to carbohydrate components (22, 23). More recently, it has been found that the cytotoxic activity of guinea pig serum on mouse thymus cells could be inhibited by a number of simple sugars (24), suggesting that natural antibodies present in the serum can react with saccharide structures on the surfaces of these cells. It has also been shown that normal human sera contain cytotoxic antibodies to neuraminidase-treated lymphocytes and that these antibodies could be inhibited by oligosaccharides (25).

Several laboratories have reported results consistent with the isolation of anti-carbohydrate antibodies. Gerisch *et al.* (7) have reported that the Fab fragments of antibodies against heated cells are univalent fragments of "anti-carbohydrate" antibodies, with specificity directed at the surface glycosphingolipid complex called "antigen I". More recently, rabbit antiserum to normal human IgM was fractionated on an affinity column to which glycopeptides of human IgM were coupled (6) and antibodies directed against the carbohydrate moiety of human IgM were separated by this procedure from the bulk of the anti-IgM antibodies. The general reactivity of these antibodies with glycoproteins was not reported, however.

The origin of natural antibodies to carbohydrate structures is not known. It has been noted that when animals are immunized with a glycoprotein such as fetuin, the elicited immune response is predominantly directed at the protein portion of the molecule and not at the carbohydrate moiety (8). The difficulty of raising antibodies to sugar portions of glycoproteins may be due to the obvious similarities (Fig. 1) in many of the carbohydrate structures appearing in otherwise unrelated serum glycoproteins such as fetuin, thyroglobulin, Ig molecules of different classes, or cell surface glycopeptides, including the histocompatibility antigens (9–11, 26, 27). The similarities of the carbohydrates may have rendered the animals tolerant to these structures, which would be recognizable as "self" antigens.

The CS-Ig fractions described here may represent products of an immune response to carbohydrate antigens on microorganisms, for example, type XIV pneumococcal polysaccharide antigen, whose immunodominant group is the sequence D-galactose-(β1 → 4)-*N*-acetyl-D-glucosamine (28), a structure closely resembling that of the sugar moiety of fetuin (9). It is also plausible that saccharide antigens are uncovered by shedding from autologous cells during their lifetime (25). Alternatively, the intriguing possibility exists that CS-Ig fractions may represent an entirely new system of antibodies directed at different groups of tissue antigens analogous to the blood group substances, Forsmann, and other heterophile antigens (29, 30).

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