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Bovine serum conglutinin is a lectin which binds non-reducing terminal *N*-acetylglucosamine, mannose and fucose residues

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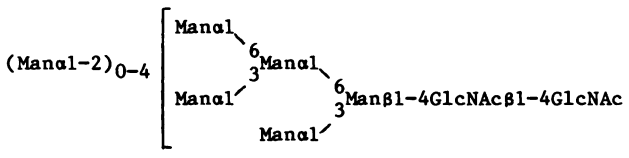
Carbohydrate recognition by bovine serum conglutinin has been investigated by inhibition and direct binding assays using glycoproteins and polysaccharides from *Saccharomyces cerevisiae* (baker's yeast), and neoglycolipids derived from *N*-acetylglucosamine oligomers, mannobiose and human milk oligosaccharides. The results clearly show that conglutinin is a lectin which binds terminal *N*-acetylglucosamine, mannose and fucose residues as found in chitobiose (GlcNAcβ1-4GlcNAc), mannobiose (Manα1-3Man) and lacto-*N*-fucopentaose II [Fucα1-4(Galβ1-3)GlcNAcβ1-3Galβ1-4Glc] respectively.

INTRODUCTION

Conglutinin is a bovine serum protein which binds in a Ca^{2+}-dependent manner to a yeast cell-wall extract, zymosan, and to the cell-surface-bound complement fragment iC3b [1–3]. The protein is composed of subunits of molecular mass 48 kDa, which contain two globular domains separated by a collagen-like region [4]; 12 or more of these subunits aggregate to form the multimeric conglutinin molecule [5].

There is evidence that conglutinin may be a carbohydrate-binding protein or lectin. The binding to both zymosan and iC3b is inhibited by the monosaccharides *N*-acetylglucosamine, mannose and L-fucose, the last two to a lesser extent [6,7]. Early fractionation studies of yeast cell walls [8] showed that agglutination of complement-coated erythrocytes by conglutinin (conglutination) was inhibited by a cell-wall mucopolysaccharide fraction [9] which contained mannose, glucosamine and some 12% (w/w) protein. A fraction that reacted strongly with conglutinin was prepared from solubilized yeast cells by precipitation with conglutinin and subsequent solubilization in EDTA. This consisted of 90% (w/w) polysaccharides and is the fraction described as conglutinogen (or 'K-gen'). Several observations have suggested that asparagine-linked oligosaccharides of high-mannose type on iC3b serve as receptors for conglutinin. Firstly, treatment of C3c (the glycosylated fragment derived from iC3b) with endo-β-*N*-acetylglucosaminidase H abolished reactivity with conglutinin [7]. Secondly, the oligosaccharide structures detected so far on human C3 have been exclusively of high-mannose type, with five to nine mannose residues [10,11]:



Thirdly, agglutination of complement-coated erythrocytes could be inhibited [12] by the disaccharide Manα1-2Man, a sequence found in the non-reducing terminal position of high-mannose-type oligosaccharides containing six to nine mannose residues. However, glycoproteins containing exclusively complex-type oligosaccharides, such as agalacto-orosomucoid, were reported also to inhibit the binding of conglutinin to mannan [13], suggesting that structures other than high-mannose-type oligosaccharides may be recognized. In order to gain further insights into the possible carbohydrate specificity of conglutinin, we have examined its reactivity with several yeast glycoconjugates and with oligosaccharides and neoglycolipids containing non-reducing terminal *N*-acetylglucosamine, mannose or fucose residues.

MATERIALS AND METHODS

Glycoproteins and oligosaccharides

A polysaccharide fraction ('K-gen') which inhibits conglutinin binding to iC3b-coated erythrocytes was isolated from *Saccharomyces cerevisiae* (baker's yeast) by digestion with a cellulase enzyme, precipitation with conglutinin and elution with EDTA, followed by digestion with trypsin [1]. Glucan from barley (*Hordeum vulgare*), and the *S. cerevisiae* products invertase (EC 3.2.1.26; grade VII), carboxypeptidase Y, glucan, mannan and zymosan A were from Sigma Chemical Co., Poole, Dorset, U.K. The milk oligosaccharides lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc), lacto-*N*-fucopentaose I (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc), lacto-*N*-fucopentaose II [Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc], lacto-*N*-fucopentaose III [Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc] and lacto-*N*-difucohexaose I [Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc] were gifts from Dr. W. M. Watkins of the Medical Research Council Clinical Research Centre.

Abbreviations used: BSA, bovine serum albumin; GVB, gelatin/veronal buffer [4 mM-sodium barbitone buffer, pH 7.2, containing 0.145 M-NaCl, 0.83 mM-$MgCl_2$, 0.25 mM-$CaCl_2$ and 0.1% (w/v) gelatin from swine skin (Sigma)]; GVB-Ca^{2+}, GVB containing 10 mM-$CaCl_2$; C3, complement component C3; C3b, the major C3 convertase product of C3; iC3b, C3b cleaved by factor I in the presence of factor H or complement receptor type 1; C3c, the major fragment of iC3b cleaved with factor I or other proteinases; MBP, mannose-binding protein.

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Oligomers of *N*-acetylglucosamine were prepared from crab shell chitin (Sigma) by hydrolysis in conc. HCl at 40 °C for 1 h, ion-exchange chromatography on Bio-Rad AG-3 (OH^-form; X4A) and AG-50W (H^+ form; X12) resins and fractionation using a Bio-Gel P-4 column. Mannobiose (Manα1-3Man), *N*-acetylglucosamine, L-fucose and mannose were from Sigma.

Conglutinin

Conglutinin was isolated [14] from heat-inactivated bovine serum and labelled [15] with ^{125}I (New England Nuclear, Stevenage, Herts., U.K.) by using 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Sigma). The labelled conglutinin had a specific radioactivity of 1.9 μCi/μg of protein and was stored at −20 °C until used.

Binding of conglutinin to iC3b-coated erythrocytes

Antibody-coated sheep erythrocytes were prepared [16] by using a rat anti-sheep monoclonal IgM antibody, and iC3b-coated cells were prepared by incubating the antibody-coated erythrocytes with rabbit serum deficient in the C6 component of complement. Erythrocytes were used as 1% (v/v) suspensions in GVB. The assay for conglutinin binding [Fig. 1 (below), inset] was adapted from that in [17]. In brief, 30 μl of GVB, 20 μl of ^{125}I-labelled conglutinin diluted in GVB-Ca^{2+}, and 20 μl of a 1% (v/v) iC3b-coated erythrocyte suspension were layered on to 200 μl of GVB containing 25% (w/v) BSA in 400 μl Eppendorf microfuge tubes and incubated at 4 °C for 2 h. Tubes were centrifuged in a Beckman Microfuge for 3 min, and erythrocyte pellets were washed once in GVB-Ca^{2+} at 4 °C. Radioactivity bound was measured in a Nuclear Enterprises 1600 γ-radiation counter. Binding of conglutinin to sheep erythrocytes or antibody-coated cells was always less than 1% of the radioactivity added; thus binding was considered to represent the interaction of conglutinin with iC3b. For inhibition assays, 10 μl of a serial dilution of inhibitor in GVB, (glycoprotein, mono- or oligo-saccharide, or neoglycolipid incorporated into liposomes [18,19]) and 20 μl of conglutinin containing 1×10^4 c.p.m. were incubated at 18 °C for 1 h before the addition of iC3b-coated erythrocytes. Inhibitory activity was expressed as hexose [20], relative to a galactose standard, or as protein [21], with BSA as a standard.

Binding of conglutinin to yeast glycoconjugates

SDS/polyacrylamide-gel electrophoresis and electrotransfer of yeast glycoconjugates on to nitrocellulose (Bio-Rad Laboratories, Watford, Herts., U.K.) was carried out essentially as described previously [22]. After transfer, strips were washed at 37 °C for 2 h in GVB containing 3% (w/v) BSA. They were then overlaid at 4 °C for 2 h with a solution of GVB-Ca^{2+} containing 5×10^5 c.p.m. of ^{125}I-labelled conglutinin/ml. After having been washed in GVB-Ca^{2+} with a final rinse in GVB-Ca^{2+} containing 0.5% Triton X-100, strips were air-dried, and autoradiography carried out at −70 °C for 48 h by using a Philips Ultra-S intensifying screen and Kodak S6 film.

Glycopeptidase F treatment of invertase grade VII

A 5 mg/ml solution of invertase (grade VII) was boiled for 3 min in 0.15 M-sodium phosphate buffer, pH 8.6, containing 1% (w/v) SDS, 1% (v/v) mercaptoethanol and 10 mM-EDTA. Nonidet P40 was added to give a 6-fold excess over the SDS concentration, and the sample was diluted in 0.15 M-sodium phosphate buffer, pH 8.6, containing 10 mM-EDTA to give an invertase concentration of 1 mg/ml. Glycopeptidase F from *Flavobacterium meningosepticum* [peptide *N*-glycohydrolase, EC 3.2.2.18; Boehringer (London) Ltd., Lewes, East Sussex, U.K.] was added to give a final concentration of 5 units/ml (1 unit will hydrolyse 1 nmol of dansylfetuin glycoprotein in 1 min at pH 7.2 and 37 °C), and the sample was incubated at 37 °C for 16 h. The digest was boiled for 5 min and stored at −20 °C.

Binding of conglutinin to neoglycolipids

Neoglycolipids [18] were prepared from monosaccharides and oligosaccharides by conjugation to the lipid dipalmitoyl phosphatidylethanolamine (Sigma). T.l.c.

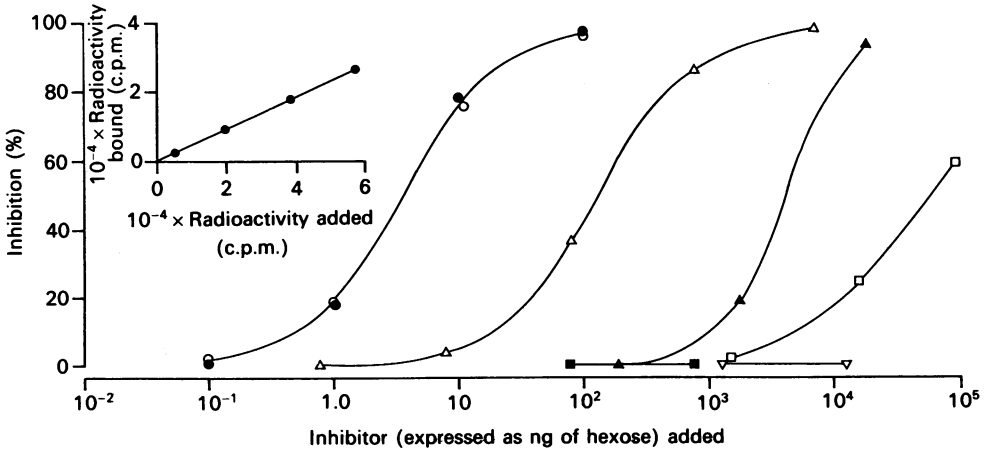

Fig. 1. Inhibition of conglutinin binding to iC3b-coated sheep erythrocytes by glycoproteins and polysaccharides

Serial dilutions of the various glycoconjugates were tested as inhibitors of the binding of ^{125}I-labelled conglutinin (1×10^4 c.p.m. added) to sheep erythrocytes coated with antibody and iC3b. ●, 'K-gen'; ○, invertase grade VII; △, zymosan A; ▲, carboxypeptidase Y; □, mannan; ■, glucan from *S. cerevisiae*; and ▽, glucan from barley. The inset shows the binding of ^{125}I-labelled conglutinin to sheep erythrocytes coated with antibody and iC3b.

and chromatogram binding assays were carried out essentially as described previously [23]. In brief, after t.l.c. on aluminium-backed silica plates, dried chromatograms were soaked in 0.05% (w/v) Plexigum P28 (Cornelius Chemical Co., Romford, Essex, U.K.) in n-hexane for 30 s, air-dried and incubated at 18 °C for 2 h in 0.85% (w/v) NaCl containing 3% (w/v) BSA. Strips were overlaid at 4 °C for 3 h with GVB-Ca^{2+} containing 5×10^5 c.p.m. of ^{125}I-labelled conglutinin/ml. After washing with GVB-Ca^{2+}, autoradiography was carried out as described above.

RESULTS

Of the glycoconjugates from *S. cerevisiae* tested as inhibitors of conglutinin binding to iC3b-coated sheep erythrocytes, 'K-gen' and the invertase preparation grade VII were by far the most active; with both materials 50% inhibition of binding was achieved with 3.7 ng of hexose added (Fig. 1). Zymosan A, carboxypeptidase Y and mannan were approx. 35, 1000 and 15500 times less inhibitory, giving 50% inhibition with 0.13, 4 and 58 μg of hexose respectively. The two samples of glucan were inactive at the highest concentrations tested (0.75 μg of hexose for glucan from *S. cerevisiae*, and 12.5 μg of hexose for glucan from barley). These latter results are in accordance with those of Lachmann & Coombs [8].

Binding assays with preparations of yeast glycoproteins and polysaccharides after polyacrylamide-gel electrophoresis and electrotransfer on to nitrocellulose (Fig. 2) showed that the predominant binding activity was associated with polydisperse high-molecular-mass components (panel 1) which were low in protein as assessed by their failure to stain with Coomassie Blue. Thus, with invertase preparation grade VII, the main Coomassie Blue-stained band, in the range 105–135 kDa (panel 2), corresponding to the invertase itself [24], showed negligible reactivity with conglutinin, demonstrating that the conglutinin bound predominantly to impurities in this enzyme preparation. Further evidence for this was obtained by subjecting invertase grade VII to column chromatography on Sephadex G-200 and monitoring the eluate fractions for inhibition of conglutinin-binding activity. The highly reactive components were in the excluded volume (results not shown). This material was found to be highly glycosylated [95% (w/w) carbohydrate measured as hexose, 5% (w/w) protein], accounting for 36% of the total hexose, but only 8% of the total protein in the grade-VII-invertase preparation.

Treatment of invertase grade VII with glycopeptidase F almost completely abolished conglutinin binding (Fig. 2, panel 2), suggesting that the recognition structures involve *N*-linked oligosaccharide chains. The reactivity of conglutinin with *N*-linked oligosaccharides of defined structure requires further investigation. However, in the present study further insights into carbohydrate recognition by conglutinin were gained by (*a*) inhibition assays using monosaccharides and oligosaccharides, and (*b*) chromatogram binding assays with neoglycolipids.

In inhibition assays, *N*-acetylglucosamine was a more potent inhibitor than mannose; 120 and 2000 nmol respectively gave 50% inhibition of binding to iC3b-coated erythrocytes. This is in accordance with previous observations in which the monosaccharides were tested as inhibitors of conglutinin binding to mannan [25], to iC3b-coated erythrocytes [6] or to purified human C3c [7]. The chitobiose sequence, which occurs in the core region of *N*-linked oligosaccharides, and the higher *N*-acetylglucosamine oligomers derived from chitin, had an activity, on a molar basis, comparable with that of the monosaccharide; 50% inhibition was given by 120, 170, 160, 150 and 125 nmol of the monomer to pentamer respectively, suggesting that non-reducing terminal *N*-acetylglucosamine is the recognition structure. The importance of multivalency for conglutinin reactivity was shown using chitobiose and chitotriose neoglycolipids assayed as liposomes. These were 400- and 550-fold respectively more inhibitory than the free oligosaccharides (0.44 and 0.30 nmol respectively gave 50% inhibition as liposomes). The neoglycolipids of *N*-acetylglucosamine assayed as liposomes were not inhibitory; this is not surprising, since the monosaccharide ring conformation is converted into an open form by conjugation to lipid [18].

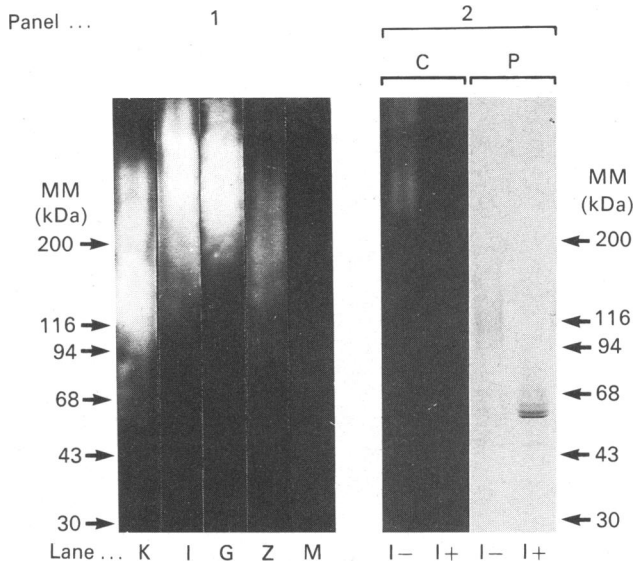

Fig. 2. Binding of ^{125}I-labelled conglutinin to glycoconjugates from *Saccharomyces cerevisiae*

Glycoconjugates were separated by polyacrylamide-gel electrophoresis in a 3–15% gel as described in the Materials and methods section. After electrotransfer on to nitrocellulose, binding of ^{125}I-labelled conglutinin was revealed by autoradiography. Panel 1: autoradiograph showing the binding of conglutinin to 'K-gen' (lane K), invertase grade VII preparation (I), high-molecular-mass component in invertase grade VII separated by Sephadex G-200 column chromatography (G), zymosan A (Z) and no detectable binding to mannan (M). None of the high-molecular-mass components bound by conglutinin showed staining with Coomassie Blue (results not shown). Panel 2: binding of conglutinin (subpanel C) and protein staining the Coomassie Blue (subpanel P) of invertase grade VII before (lanes I−) and after (lanes I+) treatment with glycopeptidase F. The amount of glycoconjugate applied was as follows: panel 1, 2 μg (expressed as hexose), except 'K-gen (0.6 μg); panel 2, subpanel C, 1 μg; subpanel P, 20 μg. Arrows indicate position of molecular-mass (MM) marker proteins: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa).

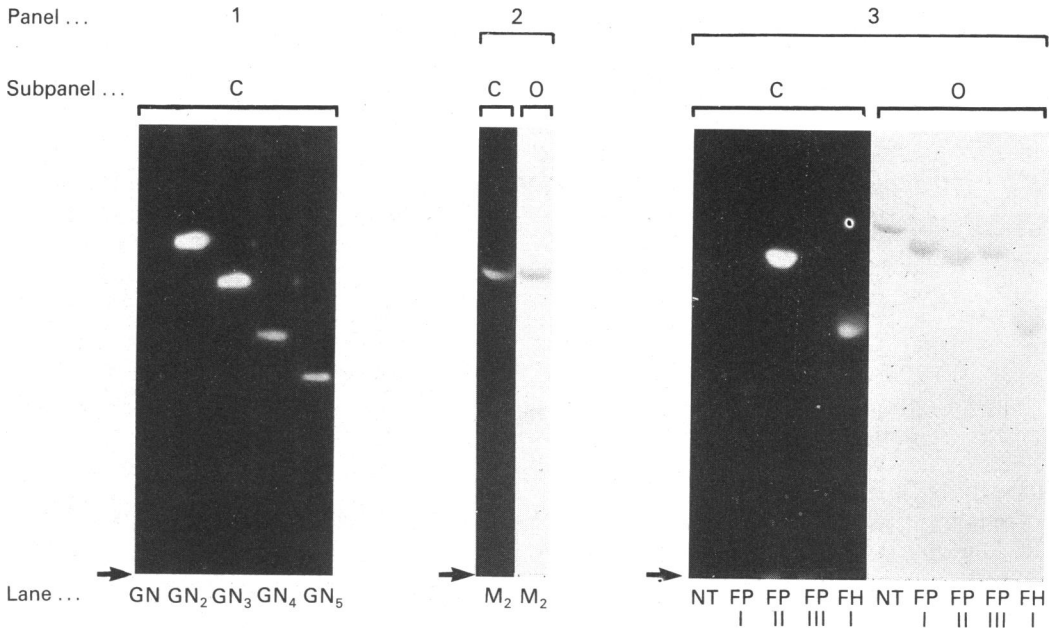

Fig. 3. Binding of ^{125}I-labelled conglutinin to neoglycolipids

The neoglycolipids were chromatographed on high-performance t.l.c. plates, and their binding to ^{125}I-labelled conglutinin revealed (in subpanels C) by autoradiography as described in the Materials and methods section. After autoradiography, neoglycolipids were revealed chemically by primulin stain for *N*-acetylglucosamine oligomers (results not shown) or by orcinol stain for neutral sugars (subpanels O). Panel 1, binding to oligomers of *N*-acetylglucosamine; panel 2, binding to mannobiose; panel 3, selective binding to milk oligosaccharides. Chromatography for panels 1 and 2 was in chloroform/methanol/water (60:35:8, by vol.), and for panel 3 in chloroform/methanol/water (105:100:28, by vol.). Arrows indicate the position of sample application. Abbreviations: GN, *N*-acetylglucosamine; GN_2–GN_5, *N*-acetylglucosamine dimer to pentamer; M_2, mannobiose; NT, lacto-*N*-tetraose; FP I, lacto-*N*-fucopentaose I; FP II, lacto-*N*-fucopentaose II; FP III, lacto-*N*-fucopentaose III; FH I, lacto-*N*-difucohexaose I. The amount of carbohydrate applied was 1 μg per lane, except for panel 1, where it was 1.6 nmol per lane.

Conglutinin binding to carbohydrate was demonstrated visually by chromatogram binding assays with neoglycolipids (Fig. 3). The neoglycolipids of the four *N*-acetylglucosamine oligomers tested (dimer to pentamer) had comparable reactivities (Fig. 3, panel 1), again showing the importance of terminal *N*-acetylglucosamine. Binding of conglutinin to mannose residues was also clearly shown when the mannobiose neoglycolipid was used as a probe (Fig. 3, panel 2), although reactivity was less intense than with the *N*-acetylglucosamine probes. The recognition of fucose residues by conglutinin was investigated in view of the known weak inhibitory activity of this monosaccharide [6]. In the present study, 23% inhibition of binding to iC3b-coated erythrocytes was given by 3000 nmol of L-fucose. However, chromatogram binding assays with neoglycolipids revealed a strong and selective reaction with lacto-*N*-fucopentaose II and lacto-*N*-difucohexaose I (Fig. 3, panel 3). Clearly this reactivity was related to the presence of the Fucα1-4GlcNAc sequence in these two oligosaccharides, since the non-fucosylated oligosaccharide lacto-*N*-tetraose and lacto-*N*-fucopentaose I and III [which contain the Fucα1-2Gal and the Fucα1-3(Galβ1-4)GlcNAc sequence respectively] failed to bind conglutinin.

DISCUSSION

The six preparations of glycoproteins or polysaccharides from *S. cerevisiae* tested as inhibitors of conglutinin binding to iC3b-coated erythrocytes may be divided into four categories on the basis of their reactivities: (i) the most active ('K-gen' and invertase preparation grade VII); (ii) intermediate (zymosan A); (iii) the least active (carboxypeptidase Y and mannan); and (iv) inactive (glucan). A common feature of the preparations with strong or intermediate inhibitory activity was the presence of polydisperse high-molecular-mass material, which bound conglutinin. With the high-molecular-mass component in the invertase preparation, binding was virtually abolished by digestion with glycopeptidase F. This suggests that, as with the complement component C3c (binding abolished after treatment with endo-β-*N*-acetylglucosaminidase H [7]), the binding of conglutinin may involve oligosaccharides *N*-glycosidically linked to protein, although recognition of a protein conformation affected by deglycosylation was not ruled out. Structural and immunochemical studies on the oligosaccharides of purified invertase [26–28], carboxypeptidase Y [29] and mannan [30,31] have shown that all three substances contain high-mannose type oligosaccharide structures based on a backbone of repeating 1-6-linked α-mannose residues, with short side chains of 1-2- and 1-3-linked α-mannose residues [29]. Most of the sequences on these substances are unlikely to be optimum recognition structures for conglutinin, since in the present study invertase itself was found to have negligible reactivity, and carboxypeptidase Y and mannan were the least active of the glycoconjugates tested. This suggests that other struc-

tures may be responsible for the superior reactivities of the 'K-gen' preparation and the high-molecular-mass components in the preparations of invertase and zymosan A.

Direct evidence that conglutinin binds to carbohydrate rather than protein was obtained by chromatogram binding assays with neoglycolipids. Although a disaccharide characterized as Manα1-3Man was reported not to inhibit the binding of conglutinin to iC3b-coated erythrocytes [12], our studies clearly show that conglutinin can bind to this structure when presented in a multivalent display. The Manα1-2Man sequence was not available for testing. The chromatogram biding assays showed that, in addition, oligosaccharides containing non-reducing terminal *N*-acetylglucosamine or the Fucα1-4(Galβ1-3)GlcNAc sequence are selectively bound by conglutinin. These findings, especially the binding to the neoglycolipid of lacto-*N*-fucopentaose II, indicate that the binding specificity of conglutinin is related to that of the Ca^{2+}-dependent macrophage receptor which mediates pinocytosis of glycoproteins [32]; the uptake of β-glucuronidase by this receptor was inhibited by the monosaccharides *N*-acetylglucosamine, mannose and fucose and by lacto-*N*-fucopentaose II, but not by the non-fucosylated oligosaccharide lacto-*N*-tetraose. In its reactivity with yeast mannan and the inhibition patterns with monosaccharides, conglutinin also resembles the mannose-binding protein (MBP) in bovine serum [25] and in the liver and sera of other mammalian species [13,33–35]. Moreover, preliminary *N*-terminal-amino-acid sequencing data indicate that there is a structural similarity between bovine conglutinin and the rat mannose-binding proteins MBP-A and MBP-C [12]. Conglutinin may therefore have been the first described member of the family of Ca^{2+}-dependent endogenous lectins molecularly characterized by Drickamer [36].

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