Cryptic I antigen activity and *Mycoplasma pneumoniae*-receptor activity associated with sialoglycoprotein GP-2 of bovine erythrocyte membranes


*Applied Immunochemistry Research Group, Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ, U.K.; and **Department of Biochemistry, Shizuoka College of Pharmacy, 2-2-l Oshika, Shizuoka-Shi, 422, Japan

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The 250-kDa sialoglycoprotein of bovine erythrocyte membranes, GP-2, has been found to be an exceptionally rich source of branched sialo-oligosaccharides of poly-N-acetyllactosamine (I antigen) type with receptor activity for the human pathogen *Mycoplasma pneumoniae*. Desialylated GP-2 is the most potent I-active substance thus far tested. Since this glycoprotein is hydrophobic and can be readily re-incorporated into cell membranes, it should be useful in future studies of the mechanism of production of auto-antibodies to the I antigen which commonly arise following human infection with *M. pneumoniae*.

The sialoglycoproteins of bovine erythrocyte membranes differ from those of human erythrocytes in the chain length of their O-glycosidically-linked oligosaccharides. With the exception of rare variants (1) the human sialoglycoproteins (glycophorins) have short oligosaccharides (2) based on the disaccharide core-region sequence Galβ1→3GalNAc as in structures 1 and 2 (Fig. 1). In contrast, long O-linked chains with poly-N-acetyllactosamine backbones, (Galβ1→4GlcNAc)_n, are characteristic of the sialoglycoproteins of bovine erythrocytes. Among the acidic oligosaccharides isolated from total sialoglycoproteins of bovine erythrocytes, the shortest were the heptasaccharide structures 3, 4, and 5 (3). Such sequences have been shown to be the major receptors on erythrocyte membranes for the human pathogen *Mycoplasma pneumoniae* (4).

A 250-kDa fraction of bovine sialoglycoproteins, termed GP-2, has been isolated recently (5,6). Sugar analysis showed (5) that this glycoprotein contains N-acetylgalactosamine, N-acetylglucosamine, galactose, and sialic acid (N-glycolylnearaminic acid 96% and N-acetylearaminic acid 4%) in the molar ratios 1.0:4.0:5.2:2.9, compatible with the presence of O-linked chains with a high content of poly-N-acetyllactosamine sequences. We report here the occurrence of potent I antigen activity, masked by acid-labile residues, in addition to *M. pneumoniae*-receptor activity associated with bovine erythrocyte
GP-2. This glycoprotein, which is hydrophobic and can be readily re-incorporated into cell membranes (6), will be useful in future studies of the pathogenesis of anti-I autoantibodies (7-9) which commonly occur following human infection with *M. pneumoniae*.

**Materials and Methods**

**Erythrocyte sialoglycoproteins**

The isolation of the 250-kDa glycoprotein GP-2 from bovine erythrocyte ghosts using lithium diiodosalicylate, phenol partitioning, and ethanol precipitation followed by sequential chromatographies on phosphocellulose and CL-4B Sepharose in the presence of SDS has been described previously (5,6). For desialylation, GP-2 (5 mg) was treated with 1-M formic acid at 100°C for 1 h, dialysed against distilled water, and lyophilized (10). Glycophorin A from erythrocyte membranes (11) was a gift of Dr. C. G. Gahmberg.

For endo-β-galactosidase treatment, the glycoproteins, 13 μg of GP-2 in 170 μl and 40 μg of glycophorin A in 160 μl of 0.14-M phosphate-buffered saline, pH 7.4, were incubated at 37°C for 16 h with 120 and 160 mU of endo-β-galactosidase, respectively, from *Bacteroides fragilis* (12); thereafter, the digestion mixtures were heated at 100°C for 5 min. Glycoproteins treated with heat-inactivated enzyme served as controls.

**Determination of I and i antigen activities**

Three anti-I antibodies, Ma, Step, and Low, which recognize different epitopes on branched poly-N-acetyllactosamine sequences
Fig. 2. Examples of carbohydrate sequences reacting with anti-I and anti-i antibodies (taken from 8, 13, 14, 16-18). Anti-I Ma recognizes the 1→4, 1→6-linked branch-point sequence as found in the core regions of O-linked oligosaccharides joined to N-acetylgalactosamine (as in structure 6 and in structure 5, Fig. 1) or in the backbone regions joined to galactose (as in structures 7 and 8); anti-I Step and Low are directed predominantly at the 1→4, 1→3-linked backbone sequence of branched poly-N-acetyllactosamine structures (underlined in structures 7 and 8). The anti-i antibodies recognize repeating N-acetyllactosamines in linear sequence.

(8, 13) and two anti-i antibodies which recognize repeating N-acetyllactosamine units (8, 13, 14) in linear sequence were used. Examples of carbohydrate sequences reacting with these antibodies are shown in Fig. 2.

The I and i antigen activities of the native and mild-acid-treated GP-2 were determined by inhibition of binding of the antibodies to I- and i-active glycoproteins in a double-antibody radioimmunoassay (15).

Inhibition of binding of M. pneumoniae to erythrocytes

The native and endo-β-galactosidase-treated GP-2 were tested as inhibitors of the binding of 51Cr-labelled human erythrocytes to sheet cultures of N. pneumoniae as described previously (4) and briefly outlined in the legend to Fig. 4.

Results

I and i antigen activities

The native GP-2 showed a strong inhibitory activity with anti-I Ma, giving 50% inhibition at a glycoprotein concentration of 10 μg/ml (Fig. 3). With anti-I Step the inhibition was weak (1700 μg/ml for 50%
Fig. 3. Inhibition of binding of anti-I antibodies Ma, Step, and Low and the anti-i antibodies Den and McC by native (▲) and mild-acid-treated (●) GP-2. The I-active glycoproteins from sheep stomachs (●) and the II-active glycoproteins from human non-secretor meconium (▲) (15) were used as reference compounds.

Inhibition); with anti-I Low there was little inhibition and with the anti-i antibodies no inhibition. However, potent inhibitory activities were revealed with all three anti-I antibodies when the mild-acid-treated GP-2 was used as inhibitor. The inhibitory activities of 0.45, 2.0, and 16 μg/ml with anti-I Ma, Step, and Low were, respectively, 3 and 4 times greater or similar to those of the reference I-active glycoprotein (sheep gastric mucin). In contrast to the I antigen activities revealed, only a negligible i activity was detected with anti-i Den and McC (Fig. 1). In separate experiments (T. Feizi and O. O. Blumenfeld, unpublished observations) human glycophorin A was found to lack I and i antigen activities.

M. pneumoniae-receptor activities

GP-2 is approximately four to six times more active than glycophorin A (per mole of sialic acid) as an inhibitor of the binding of erythrocytes to M. pneumoniae (Fig. 4). Heat treatment alone resulted in some loss of inhibitory activity of glycophorin A. Following endo-β-galactosidase treatment, the inhibitory activity of GP-2, but not that of glycophorin A, was substantially reduced.
Fig. 4. Inhibition of binding of human erythrocytes to sheet cultures of *Mycoplasma pneumoniae* by bovine sialoglycoproteins GP-2 and human glycophorin A before ( ) and after ( ) treatment with endo-β-galactosidase or with heat-inactivated endo-β-galactosidase ( ) or after heat treatment of glycophorin in the absence of endo-β-galactosidase ( ). Binding of 51Cr-labelled human erythrocytes to sheet cultures of *M. pneumoniae* (strain MY9862) in '16-x-24' multiwell plates (Costar, Cambridge, MA, U.S.A.) was performed as described previously (4). For inhibition, the sheet cultures were incubated (in triplicate) with 300 μl of serial dilutions of sialoglycoprotein in 0.14-M Tris-buffered saline, pH 7.2. Erythrocytes, 1 x 10⁶ cells containing 10 x 10⁴ c.p.m. in 300 μl, were added. Attached erythrocytes were solubilized with 0.1-M NaOH and radioactivity was counted. The counts bound were determined after subtracting the counts in control plates containing mycoplasma culture medium only. Binding in the absence of inhibitors was 4-8% of counts added. Inhibition was expressed as a percentage of the counts bound in the absence of inhibitors.

Discussion

From these studies, considerable inferences can be made concerning the carbohydrate structures of GP-2. The inhibition data with the anti-i antibodies indicate that linear sequences (as in Fig. 2) with three or more N-acetyllactosamine units are lacking or they are sparsely distributed on this glycoprotein. On the other hand, inhibition data with anti-I Ma indicate that substantial amounts of the short 1+4,-1+6-linked branch-point sequences (as in structures 5 and 6, in Figs. 1 and 2, respectively), which are known to be recognized by this antibody (8), are in an accessible state in the native GP-2. From previous knowledge (16,17,13) on the combining site of anti-I Ma, it can be inferred that these sequences are in a terminal position or possibly monosubstituted at the reducing end with α1+3-linked galactose as reported previously (18) in glycolipids of bovine erythrocyte membranes. The mild acid treatment, by removing sialic acid, rendered GP-2 the most potent I-active glycoprotein thus far tested, not only with anti-I Ma but also with anti-I Step and Low, which are
known to react with branched structures containing the repeating N-acetyllactosamine units joined to one another by 1-3 linkage (17,18,13) such as structures 7 and 8 (Fig. 2). Thus, GP-2 is unique so far among membrane glycoproteins in its abundance of long, branched carbohydrate chains analogous to those associated with mucins (reviewed in refs. 19, 20).

Since endo-β-galactosidase cannot cleave short internal Galβ1→4GlcNAc sequences when they are adjacent to a branch point (21), it can be predicted that there is an abundance of branched carbohydrate chains containing extended linear domains (susceptible to endo-β-galactosidase) as shown below and terminating with sialic acid joined by α2→3 linkage (the preferred linkage for M. pneumoniae binding [4]).

\[
\text{endo-β-galactosidase resistant} \quad \text{resistant} \quad \text{susceptible}
\]

\[
\begin{array}{c}
\text{NeuGcα2→3Galβ1→4GlcNAcβ1} \\
\text{Galβ1→4GlcNAcβ1} \\
\text{6Galβ1→4GlcNAcβ1+3Galβ1→4GlcNAc}
\end{array}
\]

Evidence for the presence of NeuGcα2→3Galβ1→ sequence in the non-reducing terminus of GP-2 has been obtained (22) from binding studies with purified anti-GM₃ (NeuGc form).

On intact human erythrocyte membranes, it has been shown (4) that only the long-chain sialo-oligosaccharides with poly-N-acetyllactosamine backbones, such as those of the polyglycosyl peptides of the anion- and sugar-transport proteins, bands 3 and 4.5 respectively, serve as receptors for M. pneumoniae. Short carbohydrate chains such as those of glycoporphin can inhibit binding but do not serve as receptors for this agent on intact erythrocyte membranes. Bovine erythrocytes bind to M. pneumoniae to the same extent as human erythrocytes (L. M. L., unpublished observations). Since adult bovine erythrocytes contain negligible amounts of glucose-transport protein (23), the band 3 and the sialoglycoprotein oligosaccharides would serve as M. pneumoniae receptors on these erythrocytes. Our observations with sialoglycoproteins of human and bovine erythrocytes illustrate an important principle, namely, that there are species differences in macromolecular carriers of carbohydrate receptors for infective agents. Thus, the complexing of adhesive molecules of infective agents to specific saccharides of host-cell membranes may give rise to differing pathobiological effects, depending on the nature of the carrier proteins of the receptor sequences in various cell types.

GP-2 has been shown to be a hydrophobic protein which can be functionally re-integrated into erythrocyte membranes and confer Sendai-virus-receptor activity to receptor-negative cells (6). Therefore, this glycoprotein is ideal for membrane reconstitution experiments and for future studies of the mechanism of production of autoantibodies with anti-I specificities (7-9) and other pathobiological effects following infection with M. pneumoniae.

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References