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Epitopes recognized by serum anti-α-galactoside antibody are present on brain glycoproteins in man

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Abstract. Naturally occurring serum IgG against terminal α-galactoside epitopes (anti-Gal), present exclusively in man, apes and old world monkeys, was used as probe for these epitopes in human brain. Human brain grey matter soluble glycoproteins enriched in α galactosyl groups by affinity chromatography on jacalin-sepharose, specifically binds to human anti-Gal in immuno dot blots. Anti-Gal recognized exclusively the terminal α galactoside epitope in human brain glycoproteins since binding was abolished by the presence of 1-0-methyl α-D-galactopyranoside as well as by pretreatment of glycoproteins with coffee bean α-galactosidase. Anti-Gal-peroxidase staining of jacalin-binding human brain glycoproteins in western immuno blots revealed mainly five anti-Gal-binding polypeptides with M_r (in kDa) of 94, 108, 180, 210 and 230 respectively. Since the presence of anti-Gal in higher animals accompanies suppression of the corresponding epitope in most tissues, apparently to maintain immunological balance, possible implications of the above observation for autoimmunity, tumor metastasis and infection are discussed.

Keywords. Anti-α-galactoside antibody; anti-Gal-peroxidase; jacalin-binding glycoproteins; autoimmunity; α-galactoside epitope; human brain glycoproteins.

1. Introduction

Anti-α-galactoside antibody (anti-Gal) is a polyclonal antibody present exclusively in the sera of man, apes and old world monkeys, constituting nearly 1 % of their serum IgG content (Galili *et al* 1984, 1988a). It recognizes terminal α-galactoside epitopes with the notable exception of the blood group B antigen (Galili *et al* 1987). Anti-Gal is believed to be produced in the above animals in response to immune stimulation by enteric bacteria that bear the α-galactosyl antigenic epitopes (Galili *et al* 1988b). Increased serum concentration of anti-Gal accompanies several kinetoplastida infections (Towbin *et al* 1987; Avila *et al* 1988) and malaria (Ravindran *et al* 1988) in man. During evolution, the presence of anti-Gal in man, apes and old world monkeys caused a concomitant loss of its corresponding antigenic epitopes, mainly terminal Ga α (1 $\longrightarrow$ 3) Gal, which is widely present in animals that do not synthesize anti-Gal (Galili *et al* 1987). Sporadic expression of the Gal α(1 $\longrightarrow$ 3) Gal epitope in man in violation of this reciprocal relationship may precipitate autoimmune disorders (Etienne-Decerf *et al* 1987), entail neoplasia (Castronovo *et al* 1989) or is characteristic of senescent cells (Galili *et al* 1984). In

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Abbreviations used: anti-Gal, Anti-α-galactoside antibody; PBS, 20 mM potassium phosphate buffer, pH 7·4, containing 150 mM NaCl; PBS-6·5, 20 mM potassum phosphate buffer, pH 65, containing 150 mM NaCl; MαGal, 1-0-methyl α-D-galactopyranoside.

the present communication we present evidence to show that several glycoproteins of normal human brain grey matter contain epitopes interacting with human serum anti-Gal and that such interactions are α-galactoside-specific as well as susceptible to α-galactosidase action on the glycoproteins. Implications of the observation for neurological disorders are discussed.

2. **Materials and methods**

Fetuin, 1-0-methyl α-D-galactopyranoside (MαGal), horse radish peroxidase (type II), 4-chloronaphthol, coffee bean α-galactosidase, soluble guar gum and yeast mannan were obtained from Sigma Chemical Company, USA. Brain tissue from healthy individuals who died of accidents was obtained within 12 h post mortem from the Forensic Medicine Department, Medical College, Thiruvananthapuram, and stored frozen at – 20°C. Human serum anti-Gal, jacalin and bovine brain*ß*-galactoside-binding lectin were prepared by procedures described by Jaison and Appukuttan (1992), Sureshkumar *et al* (1982) and Caron *et al* (1987) respectively. Conjugates of these proteins with horse radish peroxidase were prepared by glutaraldehyde cross-linking as described by Heyderman *et al* (1989) in the presence of 0·2 M inhibitory sugar (galactose for jacalin, MaGal for anti-Gal and lactose for bovine brain fi-galactoside-binding lectin) to protect the sugar-binding site. Jacalin-Sepharose containing 6 mg lectin per ml gel was prepared by the cyanogen bromide procedure (Lowe 1979).

2.1 *Preparation of human brain glycoproteins enriched in α-galactosidegroups.*

All procedures were at 4°C. Twenty gram human brain grey matter was homogenized in 100 ml PBS containing 0·2 mM phenyl methyl sulphonyl fluoride, 2 mM benzamidine hydrochloride and 2 mM EDTA in a Polytron homogenizer at top speed and centrifuged at 15,000 *g* for 30 min. From the supernatant, proteins precipitating at 70% saturation of ammonium sulphate were sedimented by centrifugation at 15,000 *g*. Pellet was re-suspended in PBS-6·5 and dialysed against the same buffer (3 changes). After a further centrifugation at 15,000 *g,* dissolved proteins were passed through 15 ml jacalin-Sepharose column equilibrated in PBS-6·5. After washing the column with PBS-6·5 till washings were protein-free, bound proteins were eluted using 0·15 M galactose in the same buffer, in 2 ml fractions. Fractions containing protein were pooled, dialysed against PBS until free from galactose and concentrated by ultrafiltration.

2.2 *Sugar specificity of anti-Gal binding to human brain glycoproteins*

Indicated amounts of glycoprotein or polysaccharide dissolved in PBS were dot-blotted on 5 mm × 5 mm nitrocellulose strips (Schleicher and Schuell). After air drying, strips were blocked by dipping overnight in 5% BSA at 25° C and then incubated for l h at 4°C in 150 µl of respective peroxidase conjugate solution (50*µ*g lectin or antibody per ml) in PBS 1% BSA in the presence or absence of 0·1 M inhibitory sugar. Where effect of α-galactosidase treatment was checked, the strip was incubated at 37° C for 45 min in 150 pl 0·5 M citrate phosphate buffer, pH

5·2, containing 1% BSA with or without the enzyme (0·4 units per ml) and then washed thrice with PBS 1% BSA before conjugate treatment as described above. After treatment with the conjugate, strips were washed thrice in PBS and transferred to peroxidase substrate solution (1 ml 0·3% 4-chloronaphthol in anhydrous methanol plus 5 ml PBS and 3 ,ul 30% H_2O_2). Positive reactions appeared as well-defined blue dots against the white nitrocellulose background.

2.3 *Detection ofanti-Gal-binding glycoproteins after electroblotting*

Jacalin-binding glycoproteins were resolved by SDS-PAGE slab gel (1·5 mm thick, 10%) according to Laemmli (1970) using a mini gel apparatus (Hoefer Scientific). One lane containing molecular weight standards and another containing glycoproteins were fixed in 50% methanol and stained with Coomassie blue. Rest of the gel containing jacalin-binding glycoproteins was electrophoretically transferred to nitrocellulose sheet according to Towbin *et al* (1979) using transfer buffer consisting of 25 mM Tris, 192 mM glycine and 15% methanol, pH 8·3, and a constant current of 0·8 mA/cm^2 for 2 h at 25° C. After blocking by successive 2 h immersions in 0·2% Tween 20 and 5% BSA, sheet was incubated for 1 h at 4° C in PBS 1% BSA containing anti-Gal-peroxidase (50 μg antibody per ml), a portion in the presence and another in absence of 0·1 M MαGal. Subsequently, strips were washed thrice with PBS and probed for bound peroxidase using 4-chloronaphthol as substrate, as described above for dot blots.

3. Results and discussion

Human serum sugar-binding proteins include, in addition to anti-Gal, the Ca^{2+}–dependent serum amyloid protein that binds the cyclic pyruvate acetal of galactose as in agarose (Hind *et al* 1985), two Ca^{2+}—dependent mannose/fucose — binding proteins (Tailor and Summerfield 1987) and a Ca^{2+}-independent mannose/N-acetyl glucosamine/mannan-binding IgG (Summerfield and Tailor 1986). The anti-Gal sample used here and prepared by affinity chromatography on immobilized galactomannan in Ca $^{2+}$-free medium containing 5 mM EDTA was free from any of these proteins as shown by agglutination inhibition data (Jaison and Appukuttan 1992) as well as by the present observation that, in contrast to guar galactomannan, yeast mannan does not bind anti-Gal (figure 1A). Jacalin binds oligosaccharides containing terminal á-linked galactose or those containing the Thomsen-Freidenreich (T) antigen [Gal β (1⟶3) GalNAc α-] (Sureshkumar *et al* 1982; Mahanta *et al* 1992). Jacalin-binding glycoproteins were selected from human brain grey matter in order to get a sample enriched in α-galactoside terminals. These glycoproteins were recognized by anti-Gal through the α-galactoside-binding site of the antibody since MáGal completely inhibited recognition (figure 1B). Further support to this conclusion was the absence of anti-Gal binding to jacalin-binding glycoproteins that had been treated with cofee bean α-galactosidase (figure 1B).

Asialofetuin is rich in terminalb- linked galactose on its N-linked oligosaccharide chains (Green *et al* 1987) and in T-antigenic groups on its O-linked oligosaccharides (Spiro and Bhoyroo 1974), but has no á-galactoside group. Binding of peroxidase-conjugated bovine brain β-galactose-binding lectin to asialofetuin was unaffected by

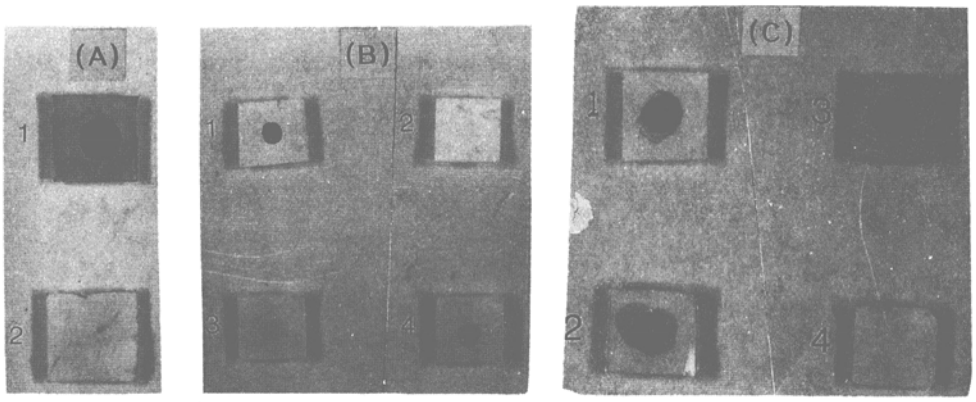

Figure 1. (A). Binding of anti-Gal-horse radish peroxidase conjugate to dot blots on nitrocellulose sheets of soluble guar gum (4 μg 1) and yeast mannan (4 μg, 2). **(B)** Anti-Gal peroxidase binding to dot blots of human brain grey matter jacalin-binding glycoproteins (2 μg each). Strips 1 and 2 were treated after blocking, with anti-Gal-peroxidase in absence (1) or presence (2) of 0·1 M MaGal. Strips 3 and 4 were incubated after blocking, in pH 5·2 buffer with (3) or without (4) coffee bean α-galactosidase before conjugate treatment. **(C)** Binding to nitrocellulose-coated asialofetuin (4 μg) of peroxidase conjugates of bovine brain β-galactoside-binding lectin (1,2), jacalin (3) and anti-Gal (4). Before conjugate treatment, strips 1 and 2 were incubated in pH 5·2 buffer in absence (1) or presence (2) of α-galactosidase. Details are in the text.

α-galactosidase treatment (figure 1C), testifying the anomer specificity of the enzyme. Asialofetuin was readily recognized by jacalin through the T-angenic groups on the glycoprotein, but not at all by anti-Gal (figure 1C). These results also indicated that the recognition of human brain glycoproteins by anti-Gal was α-galactoside specific. Probing of electroblot of jacalin-binding human brain grey matter glycoproteins resolved by SDS-PAGE, using anti-Gal-peroxidase indicated the presence of five large polypeptides containing terminal α-galactoside moiety, having M_r (in kDa) of 94, 108, 180, 210 and 230 respectively (figure 2A). Anti-Gal binding to $_{all}$ these polypeptides were inhibited by MαGal. This result also precluded the recognition of jacalin-binding human brain glycoproteins by anti-Gal being due to contamination by jacalin subunits which have M_r less than 17 kDa (Sureshkumar *et al* 1982; Mahanta *et al* 1992).

Terminal α-linked galactose groups as part of O-linked oligosaccharide side chains of glycoproteins had been reported to be present in brain tissues of many loweranimals (Finne and Krusius 1976). However since immunological balance required presence of anti-Gal and of terminal α-galactosyl epitopes to be mutually exclusive, the occurrence of these epitopes in any human tissue was unexpected (Galili *et al* 1987). Nonetheless, one explanation for the coexistence in humans of a prominent serum IgG and of brain glycoproteins with epitopes specific to this IgG, as observed here, is that these glycoproteins are in the normal course protected from exposure to the serum IgG due to sequestration of the brain cells by the blood-brain barrier. However, inflammations such as due to cancer or infections could cause breaches in the barrier (Reiber 1986) that are likely to allow an influx of anti-Gal into the brain. How far this could precipitate autoimmune disorders due to

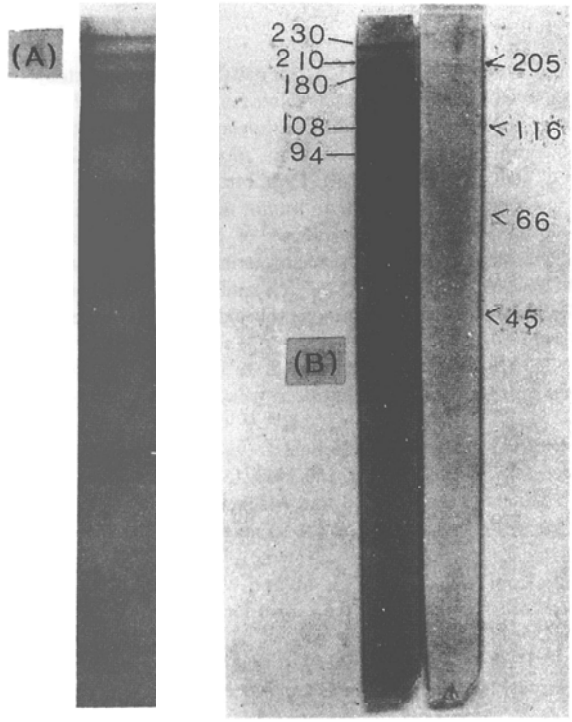

Figure 2. (A) SDS-PAGE of jacalin-binding human brain grey matter glycoproteins (40μg per well) stained with Coomassie blue. **(B)** Anti-Gal-peroxidase interaction, in absence (1) or presence (2) of 0·1 M MaGal, with the glycoproteins resolved by SDS PAGE and electroblotted to nitrocellulose sheet. Arrows indicate positions of molecular weight markers.

recognition by anti-Gal of the glycoproteins demonstrated here is a clinically important question.

Enhanced synthesis of terminal α-linked galactose groups is characteristic of many tumors (Castronovo *et al* 1989; Elices and Goldstein 1989). It has also been suggested that anti-Gal functions in an anti-tumor capacity by scavenging tumor cells that bear α-galactosyl epitopes (Castronovo *et al* 1989). The present data suggest the possibility of anti-Gal bridging between brain tissue and circulating tumor cells, thus predisposing the brain to metastasis. In view of the abundance of terminal α-galactosides in several bacteria that infect man (Jann and Jann 1978) and the recent demonstration that anti-Gal binds to them (Hamadeh *et al* 1992), a possible role for anti-Gal in localizing infectious bacteria in the central nervous system is also worth investigating.

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